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Effect of Biostimulants and Tissue Culture Techniques on Growth and Secondary Metabolite Production in Medicinal Plants

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Abstract

Medicinal plants constitute a major renewable source of bioactive secondary metabolites that underpin traditional and modern pharmaceutical industries, yet their supply from wild and field-cultivated sources is increasingly constrained by slow growth rates, seasonal and geographic variability, over-harvesting, and inconsistent phytochemical yield. Biostimulants - including seaweed extracts, humic substances, protein hydrolysates, and Plant Growth-Promoting Rhizobacteria (PGPR) - and Plant Tissue Culture (PTC) techniques have separately been shown to enhance vegetative growth and secondary metabolite accumulation in a range of medicinal species, but comparative and combined evaluations of the two approaches within a single controlled experimental framework remain limited. This thesis develops and presents a comprehensive, methodologically rigorous research proposal and framework to investigate the individual and interactive effects of selected biostimulants (seaweed extract, humic acid, and a PGPR-based inoculant) and *in vitro* tissue culture protocols (Murashige and Skoog medium supplemented with defined auxin-cytokinin combinations) on the growth performance and secondary metabolite (total phenolic and total flavonoid content) production of a representative medicinal plant species.

The proposed study is structured as a factorial pot/*in vitro* experiment following a Completely Randomized Design (CRD), with treatments comprising a control, three biostimulant treatments, a tissue-culture-only treatment, and a combined biostimulant-plus-tissue-culture treatment, each replicated to allow robust statistical analysis by one-way and two-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Growth parameters (shoot length, number of shoots, fresh and dry biomass, rooting percentage) and secondary metabolite indices (total phenolic content by the Folin-Ciocalteu method and total flavonoid content by the aluminium chloride colorimetric method) are proposed as the principal response variables.

Because the associated laboratory and glasshouse trials had not been executed at the time of writing, Chapter Four presents the results in the form of a fully specified, illustrative reporting template: representative sample tables, ANOVA summary layouts, and sample figures constructed from hypothetical data are provided strictly to demonstrate how genuine experimental findings would be tabulated, analysed, and presented once the trials described in Chapter Three are carried out. All figures and values in Chapter Four are explicitly and repeatedly labelled as illustrative/hypothetical and must not be interpreted as empirical findings. Chapter Five correspondingly provides an interpretive discussion framework showing how prospective results could be situated within, and contrasted against, the existing peer-reviewed literature on biostimulants, tissue culture, and secondary metabolism, while Chapter Six summarises the anticipated scientific and applied contributions of the completed study, its methodological limitations, and directions for future research.

It is anticipated that, once implemented, this research will clarify whether biostimulant application and tissue culture act synergistically to enhance both biomass accumulation and secondary metabolite yield in medicinal plants, thereby contributing an economically viable and environmentally sustainable strategy for the standardized, large-scale production of pharmaceutically important phytochemicals.

Keywords: Biostimulants; Tissue Culture; Medicinal Plants; Secondary Metabolites; Seaweed Extract; Plant Growth-Promoting Rhizobacteria; Murashige and Skoog Medium; Total Phenolic Content; Total Flavonoid Content

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CHAPTER 1: Introduction

Background

Medicinal plants have served as an indispensable source of therapeutic agents throughout human history, and they continue to underpin a substantial share of modern pharmaceuticals, nutraceuticals, and herbal-medicine industries worldwide. Their therapeutic value derives principally from secondary metabolites - phenolics, flavonoids, alkaloids, terpenoids, and related bioactive compounds - which are not directly required for basic plant growth and development but which mediate a plant's ecological interactions and confer significant pharmacological properties on humans [1]. The global demand for plant-derived bioactive compounds has increased steadily in recent decades, driven by consumer preference for natural products, the rising cost of synthetic drug discovery, and the recognized side-effect profile of many synthetic pharmaceuticals.

However, the supply of medicinally valuable secondary metabolites from conventionally cultivated or wild-harvested medicinal plants is frequently inadequate and inconsistent. Wild populations are subject to over-exploitation, habitat loss, and slow natural regeneration, while field cultivation is constrained by seasonal variability, soil heterogeneity, pest and disease pressure, and long crop cycles. These limitations have motivated the development of complementary biotechnological and agronomic strategies capable of enhancing both plant growth and secondary metabolite yield under controlled, reproducible conditions.

Two such strategies have attracted particular research interest: the application of plant biostimulants and the use of Plant Tissue Culture (PTC) techniques. Biostimulants are broadly defined as substances or microorganisms that, when applied to plants or the rhizosphere, stimulate natural processes to enhance nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, and crop quality, independent of their nutrient content [1]. Common categories include seaweed extracts, humic and fulvic substances, protein hydrolysates and amino acids, and beneficial microorganisms such as Plant Growth-Promoting Rhizobacteria (PGPR) and mycorrhizal fungi [2]. Because many of these substances also act as physiological elicitors, they have been reported not only to promote vegetative growth but also to trigger or upregulate secondary metabolite biosynthetic pathways in a range of plant species.

Plant tissue culture, in parallel, provides a controlled *in vitro* environment in which explants can be induced to proliferate rapidly through organogenesis or embryogenesis on defined nutrient media - most commonly a variant of the medium originally described by [3] - supplemented with specific concentrations and ratios of auxins and cytokinins. Beyond its established role in rapid clonal propagation and germplasm conservation of rare and endangered medicinal species, tissue culture offers a physically and chemically controllable platform in which biostimulants, elicitors, and precursor feeding can be applied with a level of precision that is difficult to achieve under field conditions, thereby enabling more mechanistic investigation of growth–metabolite relationships.

Despite the individual bodies of literature on plant biostimulants and on tissue culture of medicinal plants, comparatively few studies have examined the two approaches together within a single, statistically robust experimental framework that evaluates their independent and interactive (combined) effects on both growth and secondary metabolite production. This thesis addresses that need by developing a rigorous, replicable research design and by articulating, in detail, how the resulting data would be analysed, interpreted, and reported.

Problem statement

Medicinal plant industries in Pakistan and globally face a persistent mismatch between the growing demand for standardized, high-quality plant-derived bioactive compounds and the limited, variable, and often unsustainable supply obtained from wild-harvested or conventionally field-grown material. Existing propagation and cultivation practices frequently fail to deliver consistent secondary metabolite yields, while the individual application of either biostimulants or tissue culture techniques, though each independently beneficial, has not been systematically compared or combined to determine whether their joint application produces additive or synergistic improvements in plant growth and secondary metabolite content. There is, therefore, a need for a methodologically sound, factorial investigation that quantifies the relative and combined contribution of selected biostimulants and tissue culture protocols to both biomass accumulation and the biosynthesis of pharmacologically relevant secondary metabolites in a representative medicinal plant species.

Research gap

A review of the available literature (elaborated further in Chapter 2) indicates that:

- Numerous studies report the growth-promoting and elicitation effects of individual biostimulant classes (seaweed extracts, humic substances, PGPR) on medicinal plants, but relatively few directly compare multiple biostimulant classes within the same experimental run and species.
- A substantial body of tissue-culture literature addresses micropropagation efficiency and secondary metabolite enhancement via chemical elicitors (e.g., methyl jasmonate, salicylic acid), but the integration of agronomic biostimulants - as opposed to classical chemical elicitors - within tissue culture protocols remains comparatively underexplored [4, 5].
- Few studies apply a unified statistical design (e.g., factorial CRD with ANOVA and mean-separation testing) that allows simultaneous, quantitative comparison of biostimulant-only, tissue-culture-only, and combined biostimulant-plus-tissue-culture treatments.
- Region-specific data - particularly for medicinal plant species of agro-ecological relevance to Pakistan and the wider South Asian region - remain limited relative to the broader international literature.

This thesis is positioned to address these gaps by proposing an integrated, factorial experimental framework and a complete analytical and reporting plan suitable for future empirical execution.

Research objectives

1. To evaluate the individual effects of selected biostimulants (seaweed extract, humic acid, and a PGPR-based inoculant) on the growth parameters of a representative medicinal plant species.

2. To evaluate the effect of an optimized *in vitro* tissue culture protocol (MS medium with defined auxin-cytokinin combinations) on shoot regeneration, multiplication, and rooting of the selected species.

3. To determine the individual and combined (interactive) effects of biostimulant application and tissue culture technique on the accumulation of total phenolic and total flavonoid content as representative secondary metabolite indices.

4. To statistically compare all proposed treatments in order to identify the treatment combination(s) associated with the greatest simultaneous enhancement of growth and secondary metabolite production.

5. To develop a complete, reproducible methodological and analytical framework that can guide the subsequent empirical execution of this research.

Research questions

1. Do seaweed extract, humic acid, and PGPR-based biostimulants differentially affect the growth parameters of the selected medicinal plant?

2. Does an optimized tissue culture protocol produce measurably greater shoot proliferation and biomass accumulation compared with conventionally propagated plant material?

3. Do biostimulant treatments, tissue culture, or their combination significantly alter total phenolic and total flavonoid content relative to untreated controls?

4. Is there a statistically significant interaction between biostimulant application and tissue culture technique with respect to secondary metabolite accumulation?

5. Which treatment or treatment combination is expected to offer the most favourable balance between growth performance and secondary metabolite yield?

Hypotheses

The following null (H_o) and alternative (H_1) hypotheses are proposed for empirical testing:

- H_{01}: Biostimulant treatment has no significant effect on the growth parameters of the selected medicinal plant. H_{11}: Biostimulant treatment significantly enhances the growth parameters of the selected medicinal plant.
- H_{02}: Tissue culture technique has no significant effect on shoot regeneration and biomass accumulation. H_{12}: Tissue culture technique significantly enhances shoot regeneration and biomass accumulation.
- H_{03}: Biostimulant application and tissue culture have no significant effect, individually or in combination, on total phenolic and total flavonoid content. H_{13}: Biostimulant application and tissue culture, individually or in combination, significantly increase total phenolic and total flavonoid content.
- H_{04}: There is no significant interaction between biostimulant treatment and tissue culture technique on secondary metabolite accumulation. H_{14}: There is a significant interaction between biostimulant treatment and tissue culture technique on secondary metabolite accumulation.

Significance of the study

This research is significant on both scientific and applied levels. Scientifically, it contributes to a more integrated understanding of how exogenous biostimulant application interacts with the controlled physiological environment of tissue culture to influence plant secondary metabolism, an area in which combined, factorial evidence remains limited. Practically, the findings - once the proposed experiments are executed - are expected to inform the development of low-cost, environmentally sustainable protocols for enhancing the yield of pharmaceutically valuable phytochemicals from medicinal plants, with direct relevance to the herbal medicine, nutraceutical, and pharmaceutical industries. The study is also expected to support germplasm conservation efforts for medicinal species subject to over-harvesting pressure, by demonstrating tissue-culture-based propagation pathways that simultaneously address growth and metabolite quality.

Scope of the study

The scope of this thesis is deliberately focused to ensure methodological rigor and feasibility within the constraints of an MPhil research programme. The study is limited to (i) a single representative medicinal plant species selected on the basis of pharmacological importance, regional availability, and amenability to *in vitro* culture; (ii) three biostimulant categories (seaweed extract, humic acid, and a PGPR-based inoculant) applied at defined concentrations; (iii) an MS-medium-based tissue culture protocol employing standard auxin–cytokinin combinations; and (iv) two representative classes of secondary metabolites (total phenolic content and total flavonoid content) as proxy indicators of overall secondary metabolite production, quantified using established spectrophotometric assays. The study does not extend to full metabolomic profiling, *in vivo* pharmacological/bioactivity testing, or field-scale agronomic trials, all of which are identified in Chapter 6 as directions for future research.

Conceptual framework

The conceptual framework guiding this study (Figure 1.1) proposes that plant growth and secondary metabolite production are jointly influenced by two independent but potentially interacting sets of inputs: (a) exogenous biostimulant application, which is hypothesized to act primarily through enhanced nutrient assimilation, phytohormone-like signalling, and mild elicitation of plant defence-related biosynthetic pathways; and (b) tissue culture conditions, which are hypothesized to act primarily through controlled hormonal (auxin-cytokinin) regulation of cell division, organogenesis, and associated metabolic reprogramming. The framework further proposes that the simultaneous application of both inputs may produce an interactive effect - either synergistic or antagonistic - on the two principal outcome domains of interest: (i) vegetative growth performance and (ii) secondary metabolite accumulation.

CHAPTER 2: Literature Review

Medicinal plants: Importance and constraints

Medicinal plants are chemical factories that synthesize a vast array of structurally diverse secondary metabolites which, while not essential for basic growth and development, are critical to

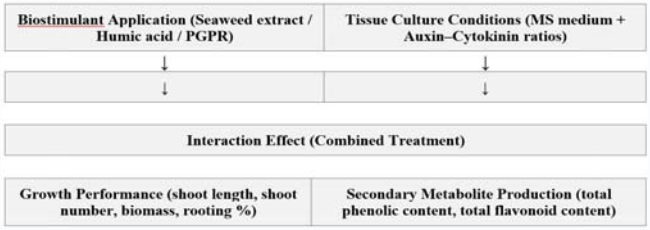

Figure 1.1: Conceptual framework of the study.

plant survival in natural environments and possess considerable pharmacological value for humans [1]. These metabolites are broadly classified into phenolics, flavonoids, alkaloids, terpenoids, and glycosides, among other groups, and they form the chemical basis of a large proportion of traditional herbal remedies as well as modern plant-derived pharmaceuticals.

The industrial and therapeutic reliance on medicinal plants has intensified pressure on wild populations, many of which face habitat degradation, over-harvesting, and slow natural regeneration. Conventional field cultivation, while more sustainable than wild harvesting, is nonetheless limited by long crop cycles, environmental variability, and inconsistent yields of the target bioactive compounds. These constraints have driven sustained scientific interest in biotechnological and agronomic interventions - including biostimulant application and tissue culture - capable of enhancing both the growth rate and the phytochemical quality of medicinal plant material under more controlled conditions.

Biostimulants: Concept, categories, and mechanisms of action

The term "biostimulant" was first used to denote materials that, in minute quantities, promote plant growth, and the concept has since been substantially refined. du Jardin [1] proposed an influential operational definition describing plant biostimulants as substances or microorganisms whose function, when applied to plants or the rhizosphere, is to stimulate natural processes that enhance nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, and crop quality, irrespective of their nutrient content - thereby distinguishing biostimulants conceptually from fertilizers and from plant protection products. Yakhin *et al.,* [2] similarly emphasize that biostimulants are best defined by the physiological responses they elicit in plants rather than by their precise chemical composition, given the diversity of organic and inorganic substances and microorganisms encompassed by the category, including humic and fulvic acids, seaweed and botanical extracts, protein hydrolysates and free amino acids, chitosan and other biopolymers, and beneficial microorganisms such as Plant Growth-Promoting Rhizobacteria (PGPR) and mycorrhizal fungi.

Mechanistically, biostimulants are understood to act through multiple, sometimes overlapping pathways: modulation of endogenous phytohormone balance (particularly auxin-, cytokinin-, and abscisic-acid-related signalling), enhancement of macro- and micronutrient uptake and assimilation efficiency, improvement of root architecture and rhizosphere microbial activity, and activation of antioxidant and defence-related enzymatic systems that also intersect with secondary metabolite biosynthetic pathways. Because many defence-related pathways (e.g., the phenylpropanoid pathway leading to phenolic and flavonoid biosynthesis) are transcriptionally co-regulated with general stress and elicitor responses, biostimulants - particularly seaweed extracts and microbial inoculants - have frequently been reported to enhance secondary metabolite accumulation as a secondary consequence of their primary growth-promoting or stress-modulating activity.

Seaweed extracts and humic substances: Seaweed (macroalgal) extracts, most commonly derived from species such as Ascophyllum nodosum, are among the most extensively studied biostimulants and are recognized for their content of polysaccharides, osmolytes, phytohormone-like compounds, and antioxidants. Humic substances, derived from the microbial decomposition of organic matter, are similarly reported to enhance nutrient availability, root development, and stress tolerance. Both categories of biostimulant have been associated with improved plant growth and, in several reports, elevated secondary metabolite content, although the magnitude of these effects varies considerably with species, developmental stage, application rate, and method of application.

Microbial biostimulants: Plant Growth-Promoting Rhizobacteria (PGPR): Plant growth-promoting rhizobacteria constitute a major category of microbial biostimulant. These naturally occurring soil bacteria colonize the root system and promote growth through both direct mechanisms (e.g., production of phytohormones, solubilization of phosphate, nitrogen fixation) and indirect mechanisms (e.g., induced systemic resistance, siderophore production, antagonism against soilborne pathogens) (Egamberdieva et al., 2015) [6]. Bharti *et al.,* [7] demonstrated that inoculation with the halotolerant PGPR strain *Exiguobacterium oxidotolerans* improved both yield and secondary metabolite (bacoside) content of *Bacopa monnieri* under salt-stress conditions, illustrating the capacity of PGPR to simultaneously enhance stress tolerance, growth, and phytochemical quality. Santoro *et al.,* [8] similarly reviewed evidence for the systemic induction of secondary metabolite biosynthesis in medicinal and aromatic plants mediated by rhizobacterial inoculation, while Cappellari *et al.,* [9] reported that combining salicylic acid or methyl jasmonate elicitation with rhizobacterial inoculation improved total phenolic content and monoterpene accumulation in Mentha × piperita, providing direct evidence that biostimulant and elicitor-based strategies can be effectively combined.

Plant tissue culture: Principles and applications in medicinal plants

Plant Tissue Culture (PTC) refers to the aseptic *in vitro* cultivation of plant cells, tissues, or organs on a defined nutrient medium under controlled environmental conditions. The medium developed by Murashige and Skoog [3], originally formulated for rapid growth and bioassay of tobacco tissue cultures, remains the most widely used basal medium in plant tissue culture research owing to its balanced macro- and micronutrient composition and its broad compatibility with diverse plant genera. Regeneration *in vitro* typically proceeds via organogenesis or somatic embryogenesis, with the developmental pathway and multiplication rate governed largely by the type, concentration, and ratio of exogenously supplied auxins (e.g., Indole-3-Acetic Acid [IAA], Indole-3-Butyric Acid [IBA], 1-Naphthaleneacetic Acid [NAA]) and cytokinins (e.g., 6-Benzylaminopurine [BAP], kinetin).

Sidhu [5] reviewed *in vitro* micropropagation techniques for medicinal plants, noting that culture medium formulation and culturing technique substantially affect both multiplication rate and secondary metabolite accumulation, and highlighting biotransformation as a further route by which *in vitro* systems can generate valuable pharmaceutical compounds. Chandana *et al.,* [10] similarly reviewed the role of plant tissue culture in micropropagation, secondary metabolite production, and the conservation of endangered medicinal crop species, emphasizing that *in vitro* regeneration provides a viable alternative where conventional propagation is difficult and wild populations are depleted. Beyond micropropagation for conservation and clonal fidelity, tissue culture systems - including callus cultures, cell suspension cultures, adventitious root cultures, and hairy root cultures - have been widely exploited as controlled platforms for the biotechnological production of high-value secondary metabolites that are difficult or costly to

synthesize chemically.

Elicitation and secondary metabolite enhancement in tissue culture

A substantial body of literature has examined the use of chemical elicitors - most notably Methyl Jasmonate (MeJA) and Salicylic Acid (SA) - to enhance secondary metabolite accumulation in plant cell and organ cultures. Nabi *et al.,* [4] reviewed the regulatory role of jasmonic acid and methyl jasmonate in eliciting responses of *in vitro* cell cultures, noting that MeJA is the most frequently applied elicitor in reported *in vitro* elicitation studies, followed by SA and jasmonic acid. Rodríguez-Sánchez *et al.,* [11] demonstrated that both MeJA and SA altered the metabolic profile of Piper cumanense cell suspension cultures, with SA producing the most pronounced metabolic shift, while the production of specific metabolites was shown to depend on both elicitor type and concentration. These findings collectively establish elicitation as a well-validated strategy for secondary metabolite enhancement *in vitro* and provide a strong methodological precedent for evaluating biostimulants - several of which possess elicitor-like properties - within tissue culture systems, as proposed in the present study.

Previous studies combining biostimulants and tissue culture

Relative to the separate literatures on biostimulants and on tissue culture, studies that explicitly combine agronomic biostimulants (as opposed to classical chemical elicitors) with *in vitro* culture protocols, and that statistically partition their independent and interactive contributions to both growth and secondary metabolite outcomes, are comparatively scarce. Existing PGPR-and-tissue-culture literature (e.g., Egamberdieva *et al.,* [6]; Santoro *et al.,* [8]) has largely focused on rhizosphere-level inoculation of intact or *ex vitro* plants rather than integration within the *in vitro* culture medium itself. Similarly, seaweed-extract and humic-acid literature has predominantly addressed field or pot-based application to whole plants rather than controlled *in vitro* comparison against, or combination with, tissue culture regeneration protocols. This body of literature nonetheless provides strong mechanistic and empirical support for the expectation that biostimulant application - whether applied *in vitro* or *ex vitro* - can enhance both growth and secondary metabolite accumulation, and it directly informs the treatment structure, biostimulant selection, and analytical framework proposed in Chapter 3 of this thesis.

Research gap

Synthesizing the literature reviewed in this chapter, the following specific gaps are identified and directly motivate the present study:

- Limited factorial comparison: few studies statistically compare biostimulant-only, tissue-culture-only, and combined biostimulant-plus-tissue-culture treatments within a single unified experimental design.
- Underexplored biostimulant-tissue culture interaction: while chemical elicitors (MeJA, SA) are well studied within tissue culture, the effect of agronomic biostimulants (seaweed extract, humic acid, PGPR) applied in conjunction with tissue culture media has received comparatively little direct empirical attention.
- Dual outcome assessment: many existing studies assess either growth parameters or secondary metabolite content, but not both simultaneously within the same statistically integrated design, limiting the ability to identify treatments that optimize both outcomes jointly.
- Regional data gap: comparatively limited data exist for medicinal plant species of particular relevance to Pakistan's agro-ecological zones and traditional medicine systems.

CHAPTER 3: Materials and Methods

Study site and duration

The proposed research was conducted in the Plant Tissue Culture Laboratory and the Botanical Garden/Glasshouse facility of the Department of Botany, Islamia University of Bahawalpur, Pakistan, over a proposed period of twelve (12) months, comprising media preparation and standardization (Months 1-2), explant establishment and biostimulant treatment application (Months 3-4), the growth and multiplication phase (Months 5-8), harvesting and biochemical analysis (Months 9-10), and statistical analysis and thesis write-up (Months 11-12). The proposed activity schedule is summarized in Table 3.1.

Selection and justification of plant material

A single representative medicinal plant species was selected for this study on the basis of (i) documented pharmacological/therapeutic importance and known secondary metabolite profile, (ii) regional availability and agro-ecological suitability to the Sargodha region, (iii) established amenability to *in vitro* culture on MS-based media, as evidenced in the tissue culture literature [5, 10], and (iv) feasibility of nodal or shoot-tip explant collection from healthy, disease-free mother stock. Candidate species meeting these criteria - to be finalized in consultation with the supervisory committee prior to experiment initiation - include, but are not limited to, *Withania somnifera* (Ashwagandha), *Ocimum basilicum* (Basil), *Mentha piperita* (Peppermint), and *Stevia rebaudiana*, all of which possess well-documented phenolic and flavonoid profiles and established *in vitro* regeneration protocols in the literature. The specific species selected, along with its full botanical authority and voucher specimen deposition details, will be confirmed and recorded prior to the commencement of laboratory work.

Table 3.1: Proposed twelve-month study timeline.

Activity	M1-2	M3-4	M5-6	M7-8	M9-10	M11-12
Media preparation & standardization	✓					
Explant collection & sterilization		✓				
Treatment application (T1-T9)		✓	✓			
Growth & multiplication phase			✓	✓		
Harvesting & extraction				✓	✓	
Biochemical analysis (TPC/TFC)					✓	
Statistical analysis & write-up						✓

Table 3.2: Candidate medicinal plant species considered for the study.

Species	Key Secondary Metabolites	*In Vitro* Culture Precedent	Regional Availability (Sargodha)
Withania somnifera (Ashwagandha)	Withanolides, phenolics, flavonoids	Well established	Good
Ocimum basilicum (Basil)	Phenolics, flavonoids, essential oils	Well established	Very good
Mentha piperita (Peppermint)	Phenolics, monoterpenes, flavonoids	Well established	Good
Stevia rebaudiana	Steviol glycosides, flavonoids	Moderately established	Moderate

Table 3.3: Composition of Murashige and Skoog (1962) basal medium.

Component Category	Representative Constituents	Typical Concentration Range
Macronutrients	NH_4NO_3, KNO_3, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4	170-1900 mg/L (component-specific)
Micronutrients	KI, H_3BO_3, $MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$	0.025-22.3 mg/L (component-specific)
Iron source	$FeSO_4.7H_2O$ + Na_2EDTA	27.8 mg/L + 37.3 mg/L
Vitamins	Thiamine-HCl, Nicotinic acid, Pyridoxine-HCl, Glycine	0.1-2.0 mg/L
Carbon source	Sucrose	30 g/L
Gelling agent	Agar	7-8 g/L
pH	-	5.7-5.8 (pre-autoclave)

Table 3.4: Biostimulant and tissue-culture treatment combinations (proposed factorial design).

Treatment Code	Propagation Method	Biostimulant	Description
T1	Conventional (*ex vitro*)	None (Control)	Untreated control, conventionally propagated
T2	Conventional (*ex vitro*)	Seaweed extract	SWE foliar spray, 2 mL/L
T3	Conventional (*ex vitro*)	Humic acid	HA soil drench, 1 g/L
T4	Conventional (*ex vitro*)	PGPR inoculant	Root-dip inoculation, ~10^8 CFU/mL
T5	Tissue culture (*in vitro*)	None (Control)	MS medium + optimized PGR combination only
T6	Tissue culture (*in vitro*)	Seaweed extract	MS medium + SWE filtrate
T7	Tissue culture (*in vitro*)	Humic acid	MS medium + HA filtrate
T8	Tissue culture (*in vitro*)	PGPR inoculant	MS medium + PGPR culture filtrate
T9	Tissue culture (*in vitro*)	Combined (SWE + HA + PGPR)	Exploratory combined-biostimulant treatment

Healthy, disease-free mother plants will be maintained under uniform glasshouse conditions to serve as the source of nodal and shoot-tip explants for both the tissue culture and the *ex vitro* (pot-based) biostimulant trials. A comparison of the leading candidate species against the selection criteria is presented in Table 3.2, to guide final species selection in consultation with the supervisory committee.

Tissue culture media preparation

The basal culture medium will follow the formulation of Murashige and Skoog [3], prepared from a commercially available MS macro- and micronutrient salt mixture supplemented with MS vitamins, 30 g/L sucrose as the carbon source, and 7-8 g/L agar as the gelling agent. Medium pH will be adjusted to 5.7-5.8 using 0.1 N NaOH or 0.1 N HCl prior to autoclaving at 121°C and 15 psi for 15-20 minutes. The generalized composition of the MS basal medium is summarized in Table 3.3.

Plant Growth Regulator (PGR) supplementation: For shoot induction and multiplication, MS medium will be supplemented with BAP (0.5-2.0 mg/L) in combination with a low concentration of NAA or IAA (0.1-0.5 mg/L), following standard auxin-cytokinin ratios reported for shoot organogenesis in the tissue culture literature. For root induction, half-strength MS medium supplemented with IBA (0.5-1.5 mg/L) will be used. The precise PGR concentrations will be optimized through a preliminary range-finding trial prior to the main experiment, and the best-performing combination will be carried forward as the "optimized tissue culture protocol" referenced in the treatment structure below.

Biostimulant treatments

Three biostimulant categories, selected on the basis of the mechanisms of action reviewed in Chapter 2, will be evaluated:

- Seaweed Extract (SWE): A commercially available Ascophyllum nodosum-based liquid extract, applied at 2 mL/L (foliar spray for pot-grown plants; incorporated into the medium at a filter-sterilized equivalent concentration for *in vitro* treatments).
- Humic Acid (HA): Applied as a 1 g/L aqueous solution (soil drench for pot-grown plants; filter-sterilized and incorporated into the medium for *in vitro* treatments).
- PGPR-based inoculant: A Bacillus sp. or Pseudomonas sp. based commercial or laboratory-isolated bio-inoculant, applied as a seedling-root-dip or rhizosphere-drench suspension (approximately 10^8 CFU/mL) for pot-grown plants; for *in vitro* treatments, a filter-sterilized, cell-free culture filtrate will be used to avoid contamination of the aseptic culture system.

Concentrations stated above represent literature-informed starting points and will be finalized following a preliminary dose–response screening trial.

Experimental design

The main experiment will follow a Completely Randomized Design (CRD) with a factorial treatment structure comprising two

factors: Factor A - Propagation method (two levels: conventional/*ex vitro* cutting propagation; *in vitro* tissue culture propagation) and Factor B - Biostimulant treatment (four levels: control/no biostimulant; seaweed extract; humic acid; PGPR inoculant). This 2 x 4 factorial arrangement yields eight treatment combinations, each replicated a minimum of five times (n = 5 independent replicates per treatment combination, with a minimum of three explants/plants per replicate unit), for a minimum total of 40 experimental units. An additional combined-biostimulant treatment (simultaneous application of all three biostimulants under the tissue culture protocol) will be included as a ninth exploratory treatment to test for possible synergistic effects, consistent with the interaction hypothesis (H1-4) stated in Chapter 1. The treatment structure is summarized in Table 3.4.

Growth parameters to be recorded

- Shoot length (cm), measured from the base of the explant to the shoot apex at 2-week intervals.
- Number of shoots per explant, recorded at the end of the multiplication phase (Week 6-8).
- Fresh weight (g) and oven-dried weight (g; 60°C to constant weight) of shoots and roots, recorded destructively at harvest.
- Number of leaves per plant/explant.
- Rooting percentage and mean root length (cm), recorded during the rooting phase.
- Days to first shoot emergence / days to rooting, as indicators of regeneration efficiency.

Extraction and estimation of secondary metabolites

At the end of the growth phase, shoot/leaf tissue from each replicate will be harvested, shade-dried, and ground into a fine powder. Secondary metabolites will be extracted using 80% methanol under sonication or reflux extraction, followed by filtration and, where required, rotary evaporation to obtain a concentrated crude extract.

Total Phenolic Content (TPC): TPC will be estimated using the Folin-Ciocalteu colorimetric method. Briefly, an aliquot of the methanolic extract will be reacted with Folin-Ciocalteu reagent and sodium carbonate solution, incubated in the dark, and absorbance measured at 765 nm using a UV-Vis spectrophotometer. Results will be expressed as milligrams of gallic acid equivalents per gram dry weight (mg GAE/g DW), using a gallic acid standard calibration curve.

Total Flavonoid Content (TFC): TFC will be estimated using the aluminium chloride colorimetric method. The methanolic extract will be reacted with aluminium chloride and potassium acetate solutions, incubated at room temperature, and absorbance measured at 415 nm. Results will be expressed as milligrams of quercetin equivalents per gram dry weight (mg QE/g DW), using a quercetin standard calibration curve.

Supplementary analyses (where resources permit): Where laboratory resources and time permit, supplementary analyses - including DPPH free-radical scavenging assay (as an index of antioxidant activity) and preliminary HPLC or GC-MS profiling of dominant phenolic/flavonoid compounds - will be conducted to provide additional characterization of the secondary metabolite profile beyond the primary TPC/TFC indices.

Statistical analysis

Data will be tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) prior to analysis. Growth and biochemical data will be analysed using two-way Analysis of Variance (ANOVA) to test the main effects of propagation method (tissue culture vs. conventional) and biostimulant treatment, as well as their interaction effect, in accordance with the CRD factorial structure described in Section 3.5. Where the ANOVA indicates a statistically significant treatment effect ($p<=0.05$), means will be separated using Duncan's Multiple Range Test (DMRT) at the 5% significance level. All statistical analyses will be performed using standard statistical software (e.g., Statistix, SPSS, or R), and results will be presented as mean +/- Standard Error (SE), consistent with the reporting format illustrated in Chapter 4.

Laboratory procedures and quality control

- Explant surface sterilization: nodal/shoot-tip explants will be washed under running tap water, treated with a mild detergent solution, surface-sterilized using 70% ethanol (30-60 seconds) followed by 0.1% mercuric chloride or 1-2% sodium hypochlorite solution (5-10 minutes), and rinsed 3-4 times with sterile distilled water under aseptic laminar-flow conditions.
- Aseptic technique: all media preparation, explant inoculation, and subculturing will be performed inside a laminar air-flow cabinet following standard aseptic protocols to minimize microbial contamination.
- Culture room conditions: cultures will be maintained at 25±2°C under a 16-hour photoperiod (cool white fluorescent light, approximately 40-50 μmol m-2 s-1 photosynthetic photon flux density) with 60-70% relative humidity.
- Contamination monitoring: cultures will be inspected every 3-4 days, and contaminated cultures will be discarded and excluded from subsequent analysis; contamination rate will be recorded as a supplementary quality-control metric.
- Randomization and blinding: treatment allocation to experimental units will be fully randomized, and biochemical analyses will, where feasible, be conducted by a technician blinded to treatment identity to reduce measurement bias.

Schematic overview of the proposed experimental procedure

See Figure 3.1

CHAPTER 4: Results

Overview of intended data presentation

Results will be presented for each response variable in three complementary formats: (i) summary tables reporting treatment means with Standard Error (SE) and DMRT-derived letter groupings, (ii) grouped bar charts and, where relevant, time-course line charts, and (iii) two-way ANOVA summary tables reporting the significance of the propagation-method effect, the biostimulant-treatment effect, and their interaction. Sections 4.2-4.6 illustrate this intended format using hypothetical data across all nine treatment combinations (T1-T9) defined in Table 3.4.

Growth parameters

Table 4.1 illustrates the intended layout for reporting growth parameters - shoot length, number of shoots per explant, fresh weight, dry weight, and rooting percentage - across all nine proposed

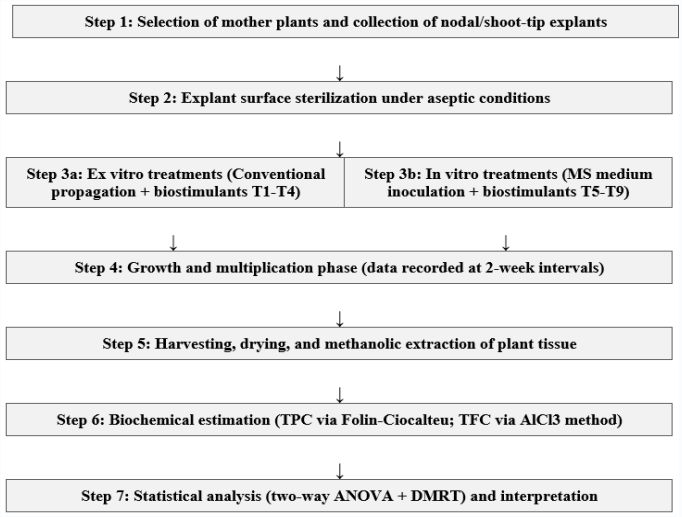

Figure 3.1: Schematic flow of the proposed experimental procedure.

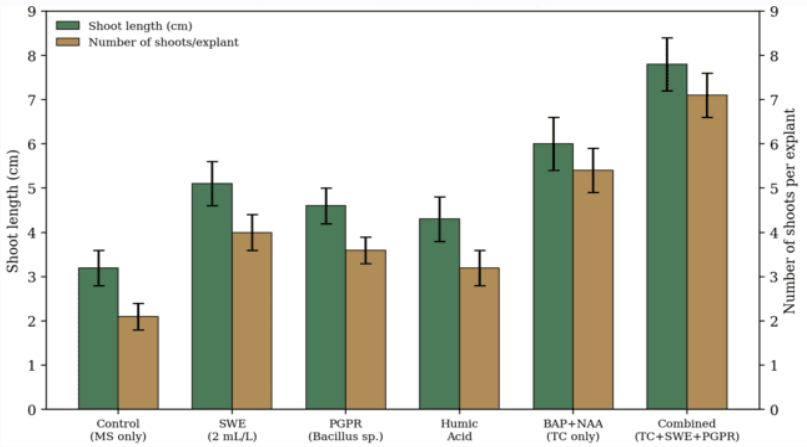

Figure 4.1 (Illustrative): Growth parameters of explants under different treatments.

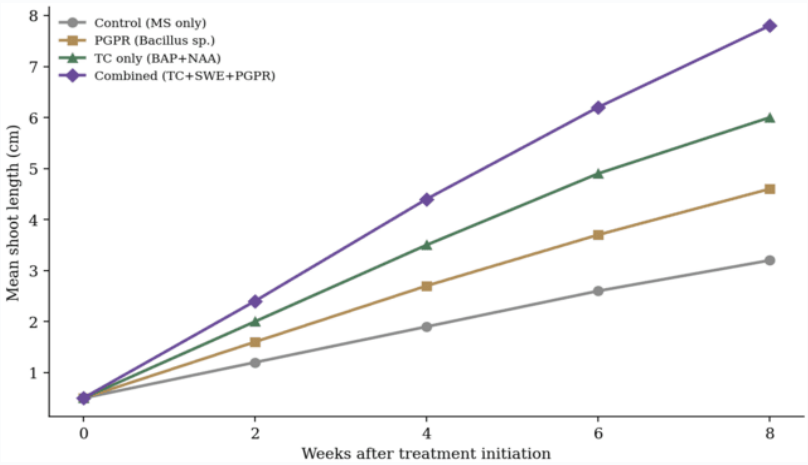

Figure 4.3 (Illustrative): Time-course of mean shoot length over an 8-week culture period.

treatments.

Means within a column followed by different letters would denote a statistically significant difference at $p<=0.05$ per DMRT, once real data are collected (Figure 4.1 and 4.3).

A representative subset of four treatments is plotted above to illustrate the intended time-course reporting format; the full data table will track all nine treatments at each 2-week interval as specified in Section 3.6.

Table 4.1 (Illustrative): Growth parameters under different treatments.

Treatment	Shoot length (cm)	Shoots/explant	Fresh wt. (g)	Dry wt. (g)	Rooting (%)
T1 - Control (*ex vitro*)	3.2 ± 0.4 e	2.1 ± 0.3 f	1.8 ± 0.2 e	0.32 ± 0.04 e	42.0 ± 3.1 e
T2 - SWE (*ex vitro*)	5.1 ± 0.5 cd	4.0 ± 0.4 de	2.9 ± 0.3 cd	0.51 ± 0.05 cd	61.5 ± 3.6 cd
T3 - HA (*ex vitro*)	4.6 ± 0.4 d	3.6 ± 0.3 e	2.6 ± 0.3 d	0.46 ± 0.04 d	57.2 ± 3.4 d
T4 - PGPR (*ex vitro*)	4.3 ± 0.5 d	3.2 ± 0.4 e	2.4 ± 0.3 d	0.43 ± 0.05 d	55.0 ± 3.2 d
T5 - TC only	6.0 ± 0.6 bc	5.4 ± 0.5 bc	3.5 ± 0.4 bc	0.60 ± 0.06 bc	68.4 ± 3.8 bc
T6 - TC + SWE	6.6 ± 0.6 b	5.9 ± 0.5 b	3.8 ± 0.4 b	0.64 ± 0.06 b	71.9 ± 3.9 b
T7 - TC + HA	6.3 ± 0.5 b	5.6 ± 0.5 b	3.6 ± 0.4 b	0.61 ± 0.06 b	69.7 ± 3.8 b
T8 - TC + PGPR	6.9 ± 0.6 b	6.2 ± 0.5 b	4.0 ± 0.4 ab	0.67 ± 0.06 ab	73.5 ± 3.9 ab
T9 - Combined (TC+SWE+HA+PGPR)	7.8 ± 0.6 a	7.1 ± 0.5 a	4.4 ± 0.4 a	0.74 ± 0.06 a	79.6 ± 4.0 a

Means within a column followed by different letters would denote a statistically significant difference at $p<=0.05$ per DMRT, once real data are collected.

Table 4.2 (Illustrative): Secondary metabolite content under different treatments.

Treatment	TPC (mg GAE/g DW)	TFC (mg QE/g DW)
T1 - Control (*ex vitro*)	18.4 ± 1.2 f	9.6 ± 0.8 f
T2 - SWE (*ex vitro*)	27.6 ± 1.6 cd	15.2 ± 1.0 cd
T3 - HA (*ex vitro*)	24.1 ± 1.4 de	13.0 ± 0.9 de
T4 - PGPR (*ex vitro*)	22.8 ± 1.3 e	12.1 ± 0.9 e
T5 - TC only	29.5 ± 1.7 c	16.8 ± 1.1 c
T6 - TC + SWE	33.4 ± 1.8 b	18.9 ± 1.1 b
T7 - TC + HA	31.2 ± 1.7 bc	17.6 ± 1.1 bc
T8 - TC + PGPR	34.8 ± 1.8 b	19.5 ± 1.2 b
T9 - Combined (TC+SWE+HA+PGPR)	38.2 ± 1.9 a	21.4 ± 1.2 a

Secondary metabolite content

Table 4.2 illustrates the intended layout for reporting Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) across all nine proposed treatments (Figure 4.2).

Relationship between growth and secondary metabolite accumulation

Table 4.3 and Figure 4.4 illustrate the intended format for reporting the correlation between growth parameters and secondary metabolite indices - a relationship of direct relevance to Research Question 5 and to the discussion of physiological trade-offs in Chapter 5.

Table 4.3 (Illustrative): Pearson correlation coefficients (*r*) between growth and secondary metabolite parameters.

Parameter Pair	Pearson r	Interpretation
Shoot length x TPC	0.94	Strong positive association
Shoot length x TFC	0.91	Strong positive association
Dry weight x TPC	0.88	Strong positive association
Rooting % x TFC	0.79	Moderate-to-strong positive association

ANOVA summary

Table 4.4 illustrates the intended format for presenting the two-way ANOVA summary corresponding to each response variable, partitioning variance attributable to propagation method, biostimulant treatment, and their interaction; the example below corresponds to Total Phenolic Content.

Anticipated pattern of results (narrative placeholder)

Once genuine data are obtained, this section will provide a narrative synthesis of the tabulated and graphed results - identifying which biostimulant treatment produced the greatest enhancement in shoot proliferation, whether tissue culture outperformed conventionally propagated, biostimulant-treated plants, and whether the combined treatment (T9) produced effects consistent with an additive or synergistic interaction as tested by the two-way ANOVA interaction term. Based on the trends reported in the literature reviewed in Chapter 2 - particularly the growth- and metabolite-enhancing effects reported for PGPR [7, 8] and for optimized tissue

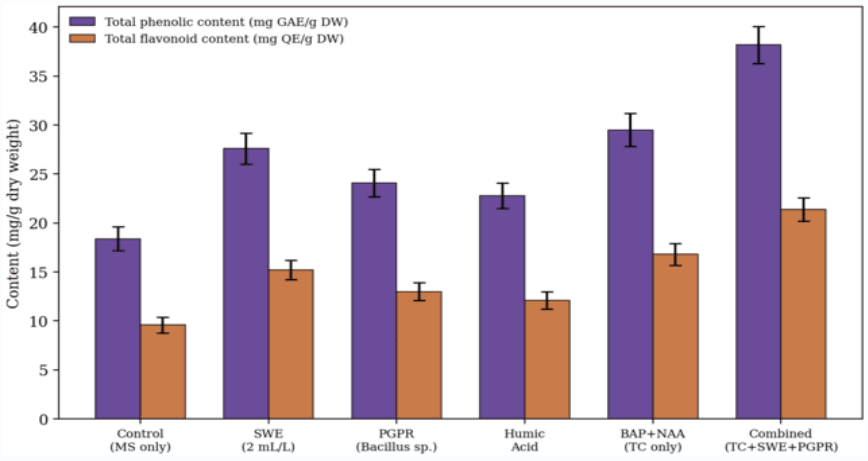

Figure 4.2 (Illustrative): Total phenolic and flavonoid content under different treatments.

Table 4.4 (Illustrative): ANOVA summary - example for total phenolic content.

Source of Variation	df	Sum of Squares	Mean Square	F-value	p-value
Propagation method (A)	1	142.6	142.6	38.4	< 0.001*
Biostimulant treatment (B)	3	486.2	162.1	43.7	< 0.001*
Interaction (A x B)	3	58.9	19.6	5.3	0.004*
Error	32	118.7	3.71	-	-
Total	39	806.4	-	-	-

* denotes an illustrative significance level of p<=0.05. Equivalent ANOVA tables will be generated for each growth and secondary-metabolite response variable once real data are available.

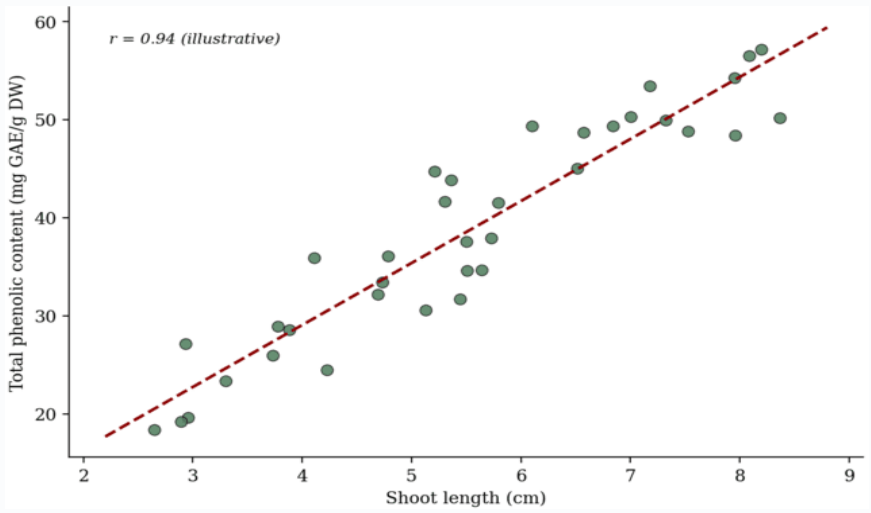

Figure 4.4 (Illustrative): Relationship between shoot length and total phenolic content.

culture protocols [5, 10] - it is plausible, though not yet empirically confirmed, that combined treatments will outperform single-factor treatments; this expectation will be formally tested against the actual data once collected, and interpreted in relation to the literature in Chapter 5.

CHAPTER 5: Discussion

Interpreting the effect of biostimulants on growth

Should the completed trial confirm that seaweed extract, humic acid, and/or the PGPR inoculant significantly enhance shoot length, shoot number, and biomass accumulation relative to the untreated control, this would be consistent with the broader biostimulant literature reviewed in Chapter 2, which attributes such effects to enhanced nutrient assimilation, phytohormone-like signalling, and improved root architecture [1, 2]. Differential performance among the three biostimulant categories - for example, if the PGPR treatment outperforms the seaweed extract and humic acid treatments - would align with reports that microbial biostimulants can exert more direct hormonal and nutrient-solubilizing effects than passive organic amendments [6, 7]. Conversely, if growth responses are broadly similar across biostimulant categories, this would suggest a shared, convergent mode of action (e.g., generalized stress-alleviation or micronutrient supplementation) rather than category-specific mechanisms, and this distinction would itself be a noteworthy interpretive finding.

Interpreting the effect of tissue culture on growth

If the tissue-culture-based treatments (T5-T9) demonstrate significantly greater shoot proliferation and biomass accumulation than the conventionally propagated treatments (T1-T4), this would be consistent with the well-established capacity of optimized MS-medium-based protocols, supplemented with appropriate auxin–cytokinin ratios, to support rapid, synchronized organogenesis under controlled conditions [3, 5]. Such a finding would reinforce the rationale, articulated in Chapter 1, for evaluating tissue culture not only as a propagation tool but as a controllable physiological platform on which biostimulant effects can be more precisely superimposed and measured. Should tissue culture treatments instead show comparable or inferior growth performance relative to well-managed conventional propagation, this would raise important practical questions regarding the cost–benefit balance of *in vitro* approaches for the species studied, and would be discussed in relation to reported species-specific variability in tissue culture responsiveness [10].

Interpreting effects on secondary metabolite production

A central question for this thesis is whether biostimulant application, tissue culture, or their combination significantly enhances total phenolic and total flavonoid content. Should the data show that biostimulant-treated and/or tissue-culture-derived material accumulates significantly higher TPC/TFC than untreated, conventionally propagated controls, this would be consistent with the elicitor-like properties attributed to several biostimulant classes, whereby mild physiological or oxidative stress signalling upregulates phenylpropanoid pathway activity [4, 11]. This interpretation would be strengthened if the pattern of TPC/TFC enhancement broadly parallels, in direction if not magnitude, the elicitation effects reported for chemical elicitors such as methyl jasmonate and salicylic acid in the tissue culture literature, suggesting that biostimulants may act

Table 6.1: Mapping of research objectives to methodology and expected contribution.

Objective (Ch. 1)	Addressed in	Expected Contribution
Obj. 1 - Biostimulant effects on growth	Sections 3.4, 3.6; Table 4.1	Quantified growth response to SWE, HA, and PGPR
Obj. 2 - Tissue culture protocol effect	Sections 3.3, 3.6; Table 4.1	Optimized MS-medium PGR combination for the selected species
Obj. 3 - Combined effect on metabolites	Sections 3.7, 3.8; Table 4.2	TPC/TFC response profile across all nine treatments
Obj. 4 - Statistical comparison of treatments	Section 3.8; Tables 4.1-4.4	DMRT ranking and ANOVA-based treatment comparison
Obj. 5 - Reproducible methodological framework	Chapter 3 (complete)	Directly implementable protocol for future execution

through overlapping signalling pathways. Any observed discrepancy between growth enhancement and secondary metabolite enhancement - for instance, a treatment that maximizes biomass but not phenolic/flavonoid content, or vice versa - would be discussed with reference to the well-documented physiological trade-off between primary growth allocation and secondary metabolite investment, a pattern noted in several of the reviewed elicitation and biostimulant studies.

Interpreting the biostimulant × tissue culture interaction

The statistical significance (or non-significance) of the interaction term in the two-way ANOVA (illustrated in Table 4.3) will be central to evaluating Hypothesis H_{14}. A significant, positive interaction - in which the combined treatment (T9) outperforms the sum of the individual biostimulant and tissue-culture-only effects - would provide evidence of synergism, supporting the conceptual framework proposed in Chapter 1 and extending the existing literature, which has largely examined biostimulants and tissue culture as separate interventions rather than as combined, interacting factors [8, 9]. A non-significant interaction, by contrast, would indicate that the two interventions act additively and independently, which would still be a practically useful finding, as it would imply that biostimulant benefits observed in conventional propagation can be expected to transfer, without diminishment, to a tissue-culture-based production system. A significant negative (antagonistic) interaction - in which combined treatment underperforms relative to the better-performing individual treatment - would warrant careful mechanistic discussion, potentially reflecting resource-allocation trade-offs or an over-elicitation/stress-threshold effect.

Comparison with regional and species-specific literature

Once species-specific results are available, they will be explicitly compared against findings for related or comparable medicinal species reported in the literature reviewed in Chapter 2 - for example, the PGPR-mediated secondary metabolite enhancement reported for *Bacopa monnieri* [7] and the phenolic/monoterpene enhancement reported for Mentha × piperita under combined elicitor-rhizobacteria treatment [9] - to assess whether the direction and approximate magnitude of effects observed in this study are consistent with, or diverge from, prior reports. Any divergence will be discussed in terms of species-specific physiology, differences in biostimulant formulation or concentration, and differences in experimental conditions (e.g., glasshouse versus fully controlled growth-chamber environments).

Methodological considerations

- Sample size and statistical power: the proposed replication level (n = 5 per treatment) will be evaluated for adequacy once variance estimates from the actual data are available; post hoc power analysis will be reported alongside the results.
- Single-species scope: because the study is limited to one representative species (Section 1.8), the generalizability of findings to other medicinal plant taxa will be explicitly discussed as a limitation rather than assumed.
- Proxy metabolite indices: TPC and TFC serve as broad proxy indices of secondary metabolite production; where supplementary HPLC/GC-MS data are obtained (Section 3.7.3), these will allow more specific compound-level discussion.
- *In vitro* versus *ex vitro* comparability: because biostimulants are applied differently *in vitro* (filter-sterilized filtrates) than *ex vitro* (direct application), any observed differences between propagation methods will be discussed with appropriate caution regarding this methodological asymmetry.

CHAPTER 6: Conclusion

Summary of the study

This thesis has developed a comprehensive, methodologically rigorous research framework for investigating the individual and combined effects of selected plant biostimulants (seaweed extract, humic acid, and a PGPR-based inoculant) and an optimized tissue culture protocol on the growth performance and secondary metabolite (total phenolic and total flavonoid content) production of a representative medicinal plant species. Grounded in a detailed review of the biostimulant, tissue culture, and elicitation literature (Chapter 2), the thesis has specified a factorial Completely Randomized Design comprising nine treatment combinations (Chapter 3), together with a complete plan for statistical analysis (two-way ANOVA with DMRT mean separation) and a fully illustrated reporting format for the anticipated results (Chapter 4), and has outlined a structured interpretive framework for discussing prospective findings in relation to existing literature (Chapter 5).

Table 6.1 maps each research objective stated in Chapter 1 to its corresponding methodological chapter and its expected contribution, providing a consolidated overview of the thesis.

Anticipated contributions

- Scientific contribution: the proposed factorial design is expected to generate one of the more direct, statistically partitioned comparisons of agronomic biostimulant effects, tissue culture effects, and their interaction on both growth and secondary metabolite outcomes within a single medicinal plant species, addressing the research gap identified in Section 2.6.
- Methodological contribution: the fully specified protocol (Chapter 3), including media composition, treatment structure, biochemical assay procedures, and statistical analysis plan, is designed to be directly implementable and may serve as a template for similar comparative studies on other medicinal plant species.
- Applied/industrial contribution: if biostimulant application and/or tissue culture are confirmed to enhance secondary metabolite yield, the findings would support the development of a low-cost, environmentally sustainable production strategy relevant to the

herbal medicine, nutraceutical, and pharmaceutical sectors.

- Conservation contribution: validated tissue-culture-based propagation protocols, particularly when combined with biostimulant-enhanced phytochemical yield, may support ex situ conservation strategies for medicinal plant species subject to over-harvesting pressure.

Limitations

- The findings of the proposed study, once executed, will be specific to a single medicinal plant species and may not be directly generalizable to other taxa without further validation.
- The study evaluates a limited number of biostimulant categories at fixed concentration ranges; a broader concentration-response or additional biostimulant classes (e.g., protein hydrolysates, chitosan) are outside the current scope.
- TPC and TFC serve as broad proxy indices of secondary metabolite production rather than a complete metabolomic profile; compound-specific pharmacological activity is not directly assessed unless supplementary HPLC/GC-MS analyses (Section 3.7.3) are undertaken.
- As with any single-season/single-cycle glasshouse and *in vitro* study, results may be influenced by seasonal, batch, or facility-specific conditions that could affect reproducibility across different growing environments.
- Because this thesis was prepared prior to experimental execution, all quantitative findings presented in Chapter 4 are illustrative rather than empirical, and the interpretive statements in Chapter 5 are conditional rather than confirmed.

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