

Optimization of Plant Growth Regulators for Enhanced Callus Formation and in Vitro Plant Growth

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Abstract: *Plant tissue culture is an important biotechnological technique for plant propagation, germplasm conservation, genetic improvement, and the production of valuable secondary metabolites. The optimization of plant growth regulators (PGRs) is a critical factor in determining the success of callus induction, callus proliferation, and subsequent in vitro plant growth. Auxins and cytokinins regulate cell division, cellular differentiation, organogenesis, and developmental responses in cultured plant tissues. The present research proposes a systematic investigation of the effects of different concentrations and combinations of plant growth regulators on callus formation and in vitro growth. Selected explants will be cultured on a suitable basal nutrient medium supplemented with varying concentrations of auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA), and cytokinins, such as 6-benzylaminopurine (BAP) and kinetin (KIN). Callus induction percentage, days to callus initiation, fresh and dry weight, callus morphology, shoot regeneration, and root development will be evaluated. The experimental data will be analyzed statistically to identify treatments associated with improved callus formation and plant growth. The proposed study is expected to contribute to the development of reproducible tissue culture protocols for plant regeneration, conservation, and future molecular investigations. The specific optimal treatment will depend on the plant species, explant type, and experimental results.*

Keywords: Plant tissue culture, callus induction, plant growth regulators, auxins, cytokinins, in vitro regeneration, plant conservation, micropropagation

I. INTRODUCTION

Plant biotechnology has become an essential component of modern agricultural science, conservation biology, and genetic research. Among the major techniques used in plant biotechnology, in vitro tissue culture provides a controlled environment for the growth and development of plant cells, tissues, and organs under aseptic conditions. This technique enables researchers to investigate plant developmental processes, produce genetically uniform planting material, preserve rare and endangered species, and establish experimental systems for genetic transformation and molecular characterization.

Callus formation represents one of the important stages in many plant tissue culture systems. A callus is an unorganized or partially organized mass of proliferating cells that develops from an explant under suitable nutritional and hormonal conditions. Callus cultures can serve as starting material for plant regeneration, somatic embryogenesis, genetic transformation, mutation studies, and the production of plant-derived metabolites. However, callus induction and proliferation vary considerably according to plant genotype, tissue type, physiological condition, culture medium, and environmental factors.



Plant growth regulators are among the most influential factors affecting the success of in vitro culture. Auxins generally promote cell expansion, cell division, and callus induction, while cytokinins stimulate cell division and influence shoot development. Nevertheless, their effects depend on concentration, interactions between different regulators, and the endogenous hormonal status of the explant. An unsuitable hormonal combination may result in poor callus initiation, excessive tissue browning, abnormal growth, or limited regeneration.

Consequently, the optimization of plant growth regulators is necessary to develop efficient, reproducible, and species-specific protocols. Rather than relying on a single concentration, systematic experimentation can establish how different hormonal treatments influence callus induction, biomass accumulation, and subsequent plant development. The present study proposes an experimental framework for identifying effective auxin–cytokinin combinations and evaluating their effects on callus formation and in vitro plant growth.

II. REVIEW OF LITERATURE

Research on plant tissue culture has established that plant cells exhibit developmental plasticity under appropriate nutritional and hormonal conditions. The following studies provide a conceptual foundation for investigating plant growth regulator optimization.

2.1 Skoog and Miller (1957): Hormonal regulation of organ formation

Skoog and Miller demonstrated the importance of the relative balance between auxins and cytokinins in regulating organ formation in cultured plant tissues. Their work established a foundational principle of plant tissue culture: the hormonal balance of the medium can influence whether cultured tissues develop roots, shoots, or proliferating tissue.

2.2 Murashige and Skoog (1962): Development of a basal nutrient medium

Murashige and Skoog developed a widely used nutrient medium for plant tissue culture. Their formulation provides mineral nutrients and other essential components that support in vitro growth. The MS medium remains a common starting point for optimizing callus induction and plant regeneration, although its suitability varies across species and explants.

2.3 George, Hall, and De Klerk (2008): Principles of plant propagation by tissue culture

George and colleagues described the principles and practical applications of plant tissue culture, including medium preparation, explant selection, hormonal regulation, callus proliferation, and plant regeneration. Their work provides a methodological foundation for designing controlled experiments in vitro.

2.4 Ikeuchi, Sugimoto, and Iwase (2013): Mechanisms of callus formation

Ikeuchi and colleagues reviewed the molecular and developmental mechanisms underlying callus formation. Their work highlighted that callus development is a regulated biological process involving hormonal signaling, gene expression, and changes in cellular identity rather than simply uncontrolled cell proliferation.

2.5 Fehér (2015): Cellular reprogramming during callus development

Fehér examined the relationship between plant cell reprogramming and callus formation. This research emphasized the importance of developmental and physiological responses in determining the capacity of plant cells to proliferate and regenerate.

Research gap: Although the general roles of auxins and cytokinins are well established, the most effective combinations are not universal. The optimal treatment must be determined for the selected plant species and explant through controlled experimentation. The proposed research will address this practical gap by comparing defined hormonal treatments using quantitative growth and regeneration parameters.

III. RESEARCH AIM AND OBJECTIVES

3.1 Aim

To optimize the concentrations and combinations of plant growth regulators for enhanced callus formation, biomass accumulation, and in vitro plant growth in a selected plant species.



3.2 Objectives

To evaluate the effects of different concentrations of auxins on callus induction and proliferation.

To determine the influence of cytokinins and auxin–cytokinin combinations on callus development and plant regeneration.

To assess the effects of hormonal treatments on callus initiation time, fresh weight, dry weight, and morphological characteristics.

To identify effective plant growth regulator combinations for shoot multiplication and root development.

To develop a reproducible in vitro culture protocol that can support plant propagation, conservation, and further molecular analysis.

3.3 Research Questions

How do different auxin concentrations affect callus induction in the selected plant species?

Which auxin–cytokinin combinations produce the most favorable callus growth?

How does hormonal composition influence shoot regeneration and root formation?

Are the observed differences between hormonal treatments statistically significant?

Can the optimized protocol be applied consistently across independent experimental batches?

IV. MATERIALS AND METHODS

4.1 Research Design

The study will use a controlled laboratory experiment to evaluate the effects of plant growth regulators on callus formation and in vitro growth. A completely randomized design (CRD) is proposed for the initial optimization experiment, subject to laboratory conditions and the availability of sufficient culture vessels.

The plant species, explant type, basal medium, and treatment concentrations will be selected based on preliminary trials and existing species-specific literature. Young leaves, nodal segments, hypocotyls, cotyledons, or other suitable tissues may be considered depending on the biological characteristics of the selected species.

Each hormonal treatment should include independent biological replicates, with the final sample size determined through preliminary variability estimates or a power analysis. Culture vessels should be randomized within the growth environment to reduce the influence of positional effects.

4.2 Collection and Preparation of Explants

Healthy, disease-free donor plants will be selected as the source of explants. Plant material will be collected from appropriately identified specimens and transported to the laboratory under suitable conditions.

The explants will be washed to remove soil and surface contaminants. Surface sterilization will be performed using a validated procedure appropriate to the plant species and tissue type. A suitable sterilant, exposure time, and number of sterile-water rinses will be established through preliminary trials to balance microbial control against tissue injury.

Sterilized explants will be trimmed using sterile instruments and inoculated onto the designated culture medium under aseptic conditions. Contamination frequency and explant survival will be recorded for each treatment.

4.3 Preparation of Culture Medium

Murashige and Skoog (MS) medium will be considered as the basal nutrient medium. The medium will contain appropriate concentrations of mineral salts, vitamins, a carbon source such as sucrose, and a gelling agent when solid culture is required.

The pH will be adjusted before sterilization according to the established protocol for the selected species. Media and culture vessels will be sterilized using a validated laboratory procedure.

Plant growth regulators will be added at suitable concentrations using a consistent preparation procedure. Where required, stock solutions will be prepared and stored according to the stability and handling requirements of each regulator.

4.4 Selection of Plant Growth Regulators

The study will investigate representative auxins and cytokinins.



Plant growth regulator	Type	Proposed function
2,4-D	Synthetic auxin	Induction and maintenance of callus in responsive tissues
NAA	Synthetic auxin	Callus induction, cell proliferation, and root-related responses
IBA	Auxin	Root initiation and development in suitable regeneration systems
BAP	Synthetic cytokinin	Shoot initiation and multiplication
Kinetin (KIN)	Synthetic cytokinin	Cell division and shoot development in responsive tissues

Not every regulator needs to be included in every experimental stage. The selection will depend on the species, previous findings, and preliminary responses.

4.5 Experimental Treatments for Callus Induction

An initial screening experiment will compare a control treatment with selected auxin concentrations and auxin–cytokinin combinations. The following table provides an illustrative treatment structure, not a universally validated formulation.

Treatment	Illustrative hormonal condition	Purpose
T0	Basal medium without added PGRs	Control
T1	Low concentration of selected auxin	Assess initial callus response
T2	Intermediate concentration of selected auxin	Assess callus induction and proliferation
T3	Higher concentration of selected auxin	Evaluate concentration-dependent effects
T4	Selected auxin + low BAP	Examine auxin–cytokinin interaction
T5	Selected auxin + intermediate BAP	Compare callus induction and tissue development
T6	Selected auxin + kinetin	Evaluate an alternative cytokinin combination

Exact concentrations, exposure durations, and the number of treatments must be established for the selected plant species using relevant published protocols and preliminary experiments. The screening stage may be followed by a second experiment that refines the most promising concentration ranges.

4.6 Callus Induction and Proliferation

Inoculated explants will be incubated under controlled temperature, light, and photoperiod conditions suitable for the selected species. The incubation period will be determined from preliminary observations and published protocols. Cultures will be examined at predefined intervals for callus initiation, tissue color, texture, approximate coverage of the explant, contamination, and signs of necrosis. Callus formation will be recorded using a consistent scoring system. After the induction stage, callus tissue may be transferred to fresh medium containing the selected hormonal treatments to assess proliferation. The transfer interval will be standardized across treatments.

Callus color and texture will be documented photographically. Morphological observations may include compact, friable, nodular, pale, green, or browning tissue, where applicable. These characteristics will be interpreted cautiously because appearance alone does not establish regenerative capacity or genetic stability.

4.7 Assessment of In Vitro Plant Growth

Treatments showing satisfactory callus induction will be selected for further investigation of regeneration. Depending on the species, this stage may involve transfer to a shoot-induction medium containing a suitable cytokinin, followed by transfer to a rooting medium containing an appropriate auxin or to a regulator-free medium.

The following parameters will be assessed:

Percentage of explants forming callus.

Number of days to visible callus initiation.

Fresh weight and dry weight of callus.

Callus morphology and degree of browning.



Percentage of cultures producing shoots.

Number and length of regenerated shoots.

Percentage of shoots forming roots.

Root number and root length.

Survival and growth of regenerated plantlets during acclimatization.

Shoot and root development will be measured at standardized intervals. Plantlets will be transferred to an appropriate acclimatization environment only after sufficient development, and survival will be monitored over a predefined period.

4.8 Data Collection and Quantitative Measurements

The principal response variables will be calculated using standardized formulas.

Callus induction percentage

$$\text{Callus induction (\%)} = \frac{\text{Number of explants forming callus}}{\text{Total number of viable explants cultured}} \times 100$$

Shoot regeneration percentage

$$\text{Shoot regeneration (\%)} = \frac{\text{Number of explants producing shoots}}{\text{Total number of viable explants assessed}} \times 100$$

Mean fresh weight

$$\bar{W}_f = \frac{\sum_{i=1}^n W_{fi}}{n}$$

where W_{fi} represents the fresh weight of the (i)-th independent experimental unit.

Relative callus growth

Where initial and final measurements are available, relative growth may be expressed as:

$$\text{Relative growth} = \frac{W_f - W_i}{W_i}$$

where (W_i) and (W_f) denote initial and final callus weights, respectively. This measure should only be used when the initial mass is nonzero and measured consistently.

Dry weight will be determined using a standardized drying procedure until a predefined constant-weight criterion is reached. The experimental unit, whether an explant or a culture vessel, must be clearly defined to avoid pseudoreplication.

4.9 Statistical Analysis

The data will be organized and analyzed using suitable statistical software, such as R, SPSS, or an equivalent validated package.

Continuous outcomes, including fresh weight, dry weight, shoot number, and shoot length, may be compared using analysis of variance (ANOVA) when its assumptions are reasonably satisfied. Post-hoc comparisons, such as Tukey's honestly significant difference test, may be used to identify differences among treatment means.

Callus induction and shoot regeneration percentages may be analyzed using binomial generalized linear models or other suitable methods for proportional data. If the study evaluates multiple auxin and cytokinin concentrations in a factorial design, the analysis will estimate their individual effects and interaction.

Statistical significance will be evaluated using a prespecified significance level, commonly $p < 0.05$. Effect sizes, confidence intervals, and variability estimates should also be reported. Where assumptions are not met, appropriate transformations or alternative statistical models will be considered.

The optimal treatment will be identified using predefined criteria that consider callus induction, biomass production, tissue quality, regeneration efficiency, and plantlet survival rather than relying exclusively on the highest callus induction percentage.



V. RESULTS AND DISCUSSION: PROPOSED REPORTING FRAMEWORK

Important: Since no experimental dataset has been provided, the following section describes how results should be presented and interpreted. It does not claim that experiments have been conducted or that any treatment has produced a measured improvement.

5.1 Effect of Plant Growth Regulators on Callus Induction

The first stage of analysis will compare callus induction percentages across all treatments. Differences in callus initiation time and the proportion of responsive explants will be examined to determine whether the hormonal treatments influence the initiation of proliferating tissue.

A higher callus induction percentage may indicate that a treatment supports the initiation of callus in the selected species. However, callus induction should be interpreted alongside contamination, explant survival, tissue morphology, and subsequent growth.

5.2 Influence of Auxin Concentration on Callus Proliferation

The effects of auxin concentration will be assessed using fresh weight, dry weight, and morphological observations. The analysis will determine whether the response increases consistently with concentration or whether an intermediate concentration provides a more favorable outcome.

Excessive or unsuitable auxin exposure may produce undesirable tissue responses or reduce subsequent regeneration in some species. Therefore, the best treatment will be determined empirically rather than assuming that a higher concentration is always more effective.

5.3 Interaction Between Auxins and Cytokinins

The auxin–cytokinin interaction will be examined to identify combinations associated with callus proliferation and subsequent organ formation. The relative concentrations of these regulators can influence developmental responses, but the outcome depends on the plant species and experimental conditions.

A factorial analysis can help distinguish the effect of each regulator from the effect of their interaction. This approach will provide stronger evidence than comparing isolated treatment means alone.

5.4 Callus Quality and Regeneration Potential

Callus quality will be assessed using morphological observations and the ability of cultures to produce shoots or somatic embryos, where relevant. Friable or compact callus should not automatically be classified as superior; its suitability depends on the research objective and the plant species.

Where the purpose is conservation or micropropagation, regeneration capacity and the survival of regenerated plantlets are particularly important. Where the purpose is molecular analysis or metabolite production, additional measures may be needed to establish the suitability of the callus.

5.5 Implications for Plant Conservation

An optimized tissue culture protocol could support the multiplication and preservation of valuable plant material, especially where conventional propagation is slow or limited by seed availability. Nevertheless, successful callus induction alone does not establish an effective conservation method.

For conservation applications, the study should also evaluate regeneration efficiency, acclimatization success, and genetic fidelity. Molecular markers or other validated genetic analyses may be incorporated in a subsequent phase to examine whether regenerated plants retain the expected genetic characteristics of the source material.

VI. SIGNIFICANCE OF THE STUDY

The research will contribute to plant biotechnology by examining how hormonal conditions influence the development of cultured plant tissues. A systematic optimization strategy can reduce reliance on trial-and-error procedures and provide a transparent basis for selecting culture conditions.

The study may also establish a foundation for future investigations involving somatic embryogenesis, genetic transformation, secondary metabolite production, and molecular characterization. For plant conservation, the ability to



regenerate healthy plants from selected tissues could facilitate the preservation and multiplication of important plant resources.

The broader significance of the work will depend on the reproducibility of the optimized protocol, the quality of regenerated plants, and its suitability for the selected species and intended application.

VII. LIMITATIONS OF THE STUDY

The response to plant growth regulators varies among species, genotypes, explant types, and donor plant conditions. A protocol optimized for one species may not be directly applicable to another. Contamination, tissue browning, phenolic oxidation, and variation in explant physiological age may also influence experimental outcomes.

Furthermore, a high callus induction rate does not necessarily correspond to high regeneration efficiency. Prolonged culture and repeated subculturing may introduce somaclonal variation in some plant systems. Therefore, additional genetic fidelity assessments may be required before regenerated plants are used for conservation or commercial propagation.

The conclusions will be limited to the plant material, culture conditions, treatments, and experimental design evaluated in the study.

VIII. CONCLUSION

The optimization of plant growth regulators is a central component of developing efficient plant tissue culture protocols. Auxins and cytokinins influence callus induction, proliferation, and organ development, but their effects depend on the biological characteristics of the plant and the conditions under which cultures are maintained.

The proposed research will systematically evaluate selected hormonal treatments and quantify their effects on callus induction, biomass accumulation, shoot regeneration, and root development. By combining controlled experiments with appropriate statistical analysis, the study aims to identify effective treatment combinations and develop a reproducible protocol for the selected plant species.

The resulting protocol may contribute to plant propagation, germplasm conservation, and subsequent molecular investigations. Its effectiveness, however, must be established through experimental validation, independent replication, and assessment of the quality and genetic stability of regenerated plants.

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