

Comparative Enhancement of Glycyrrhizin Content in Callus Cultures of *Achras sapota* L. and *Taverniera cuneifolia* (Roth) Arn. Through Optimization of Plant Growth Regulator Combinations

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ABSTRACT

The present investigation was carried out to evaluate the effects of different combinations of plant growth regulators (PGRs) on callus induction, biomass accumulation, and glycyrrhizin production in *Achras sapota* L. and *Taverniera cuneifolia* (Roth) Arn. Stem explants were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of α -naphthaleneacetic acid (NAA), Kinetin (Kn), 6-benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D). Callus cultures were maintained for eight weeks under controlled photoperiodic conditions and analyzed for glycyrrhizin content using High-Performance Liquid Chromatography (HPLC). Among the six tested treatments, the ternary combination of NAA (1.0 mg/L) + Kn (1.0 mg/L) + BAP (2.0 mg/L) (T6) produced the highest callogenesis frequency and glycyrrhizin accumulation. Maximum glycyrrhizin content of 6.42 mg/g dry weight (DW) and 9.84 mg/g DW was observed in *Achras sapota* and *Taverniera cuneifolia*, respectively, under T6—representing 3.13-fold and 2.64-fold enhancement over field-grown plants. The study demonstrates that strategic optimization of auxin–cytokinin balance significantly enhances secondary metabolite production in vitro, offering a sustainable biotechnological approach for commercial-scale glycyrrhizin biosynthesis.

Keywords: *Achras sapota*; *Taverniera cuneifolia*; Glycyrrhizin; Callus Culture; Plant Growth Regulators; HPLC; Secondary Metabolites; Tissue Culture.

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1. INTRODUCTION

Plant-derived secondary metabolites constitute a vast reservoir of pharmacologically active compounds with profound therapeutic relevance. Among these, glycyrrhizin (glycyrrhizic acid), an oleanane-type pentacyclic triterpenoid saponin biosynthetically derived from β -amyrin via the mevalonate pathway, is one of the most commercially important plant-derived bioactive substances. Glycyrrhizin possesses a diverse spectrum of biological activities, including anti-inflammatory, antiviral, hepatoprotective, immunomodulatory, anti-allergic, and antioxidant properties [1, 2]. It is widely used as a natural sweetener, flavoring agent, and pharmaceutical excipient in formulations intended to manage hepatitis B and C, peptic ulcers, adrenal insufficiency, and atopic dermatitis [3].

Conventionally, glycyrrhizin is extracted from the roots of *Glycyrrhiza glabra* L. (licorice) and *Glycyrrhiza uralensis*. However, overexploitation of natural licorice populations, coupled with agronomic constraints and the slow growth of root-yielding species, has necessitated the exploration of alternative, sustainable production platforms [4, 5]. *Taverniera cuneifolia* (Roth) Arn., a member of the Fabaceae family commonly distributed across semi-arid regions of India, has been identified as a promising alternative source of glycyrrhizic acid and has demonstrated successful in vitro biosynthesis of

glycyrrhizin in organized root cultures [6, 7]. *Achras sapota* L. (*Manilkara zapota*), a tropical tree of the Sapotaceae family, has also been reported to contain triterpenoid compounds and represents a candidate for callus-based secondary metabolite production [8].

Plant tissue culture methodologies, particularly callus culture, offer a controlled, scalable, and season-independent system for the production of high-value phytochemicals [9, 10]. The induction of callus and subsequent secondary metabolite accumulation are critically dependent upon the type, concentration, and ratio of phytohormones incorporated into the basal nutrient medium [11]. Auxins and cytokinins, the principal classes of plant growth regulators (PGRs), modulate cell dedifferentiation, proliferation, and biosynthetic enzyme activity governing secondary metabolic pathways [12, 13]. NAA (a synthetic auxin) and BAP or Kinetin (cytokinins) are routinely employed in combination to achieve efficient callogenesis [14]. Despite the considerable body of literature on *Glycyrrhiza*-derived glycyrrhizin, systematic comparative investigations into callus-mediated glycyrrhizin biosynthesis in alternative host plants remain sparse. The present study was, therefore, designed to systematically evaluate the effect of different PGR combinations on callus induction frequency, callus morphology, biomass accumulation, and glycyrrhizin content in callus cultures of *Achras*

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sapota and *Taverniera cuneifolia* under standardized in vitro conditions, using quantitative HPLC analysis as a robust analytical tool [15].

2. MATERIALS AND METHODS

2.1 Plant Material and Collection

Seeds and young stem explants of *Achras sapota* L. and *Taverniera cuneifolia* (Roth) Arn. were collected from the Medicinal Plant Garden, Akola, Maharashtra, India. The botanical identity of the plant specimens was authenticated by a qualified plant taxonomist, and voucher specimens were deposited with identification number Bot./H/8904. Healthy, disease-free, actively growing stem segments (1.5–2.0 cm in length) bearing at least one node were selected as explants for all experiments [16].

2.2 Surface Sterilization

Explants were thoroughly washed under running tap water for 20 minutes to remove surface debris. They were subsequently treated with 0.1% (w/v) Bavistin (carbendazim) solution for 10 minutes as a prophylactic fungicidal treatment. After rinsing twice with sterile distilled water, explants were surface-sterilized with 70% (v/v) ethanol for 30 seconds, then with a 0.1% (w/v) mercuric chloride (HgCl₂) solution for 3 minutes under aseptic conditions in a laminar airflow cabinet. Residual sterilant was removed by washing three to four times with sterile distilled water before inoculation onto culture medium [17].

2.3 Culture Medium Preparation

Murashige and Skoog (MS) basal medium supplemented with 3% (w/v) sucrose as a carbon source and solidified with 0.8% (w/v) agar (pH adjusted to 5.8 ± 0.1 before autoclaving) was used throughout the study. The medium was autoclaved at 121°C at 15 psi for 15 minutes. All filter-sterilized PGR stock solutions were added aseptically to the cooled medium (below 50°C) before dispensing into culture vessels [18].

2.4 Hormonal Treatments

Six PGR treatment combinations were investigated in the present study. Each treatment was evaluated in triplicate (n = 15 explants per treatment per species):

T1: NAA (0.5 mg/L) + Kn (0.5 mg/L)

T2: NAA (1.0 mg/L) + Kn (1.0 mg/L)

T3: NAA (1.0 mg/L) + BAP (2.0 mg/L)

T4: 2,4-D (1.0 mg/L)

T5: 2,4-D (1.0 mg/L) + BAP (2.0 mg/L)

T6: NAA (1.0 mg/L) + Kn (1.0 mg/L) + BAP (2.0 mg/L)

Cultures were maintained at 25 ± 2°C under a 16-hour photoperiod (cool white fluorescent lamps, 2500 lux) for eight weeks. Callus initiation was recorded as the number of days required for visible callus emergence, and callogenesis frequency was expressed as the percentage of responsive explants.

2.5 Extraction and HPLC Analysis of Glycyrrhizin

Eight-week-old callus was harvested, lyophilized, and pulverized. Accurately weighed dry powder (100 mg) was extracted with 80% methanol (10 mL) under sonication for 30 minutes, centrifuged at 5000 rpm for 15 minutes, and the supernatant was filtered through a 0.45-µm PTFE membrane filter. Glycyrrhizin content was determined by reverse-phase HPLC (Shimadzu LC-20AD) using a C18 analytical column (250 × 4.6 mm, 5

µm) with acetonitrile: 0.05% trifluoroacetic acid (40:60, v/v) as mobile phase at a flow rate of 1.0 mL/min and UV detection at 254 nm. Calibration was performed using authentic glycyrrhizin standard (Sigma-Aldrich, ≥98% purity). Results were expressed as mg glycyrrhizin per gram dry weight (mg/g DW) [15, 19, 20].

2.6 Statistical Analysis

All experiments were performed in triplicate and data are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine significant differences among treatments. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 21.0 (IBM, USA) [21].

3. RESULTS

3.1 Effect of PGR Combinations on Callogenesis

Callus induction was observed across all tested treatments in both plant species, though at varying frequencies and with differing morphological characteristics. *Taverniera cuneifolia* consistently exhibited higher callogenesis frequency and earlier callus initiation than *Achras sapota* across all PGR combinations, suggesting a greater inherent in vitro responsiveness in this species. The ternary combination T6 [NAA (1.0 mg/L) + Kn (1.0 mg/L) + BAP (2.0 mg/L)] was identified as the most effective treatment, producing the highest callogenesis frequency of 82% in *Achras sapota* (initiation: 9–11 days) and 90% in *Taverniera cuneifolia* (initiation: 8–10 days). The callus induced under T6 was profuse, friable, and rapidly proliferating with a creamy-white appearance—morphological characteristics indicative of high biosynthetic competency [22, 23]. The detailed callogenesis data for all treatments are presented in Table 1.

Table 1. Effect of Different PGR Combinations on Callogenesis in *Achras sapota* and *Taverniera cuneifolia*

| Treatment | PGR Combination (mg/L) | Days to Callus Initiation (<i>A. sapota</i>) | Callogenesis % (<i>A. sapota</i>) | Days to Callus Initiation (<i>T. cuneifolia</i>) | Callogenesis % (<i>T. cuneifolia</i>) | Callus Characteristics |
|-----------|------------------------|--|-------------------------------------|--|---|------------------------------|
| T1 | NAA (0.5) + Kn (0.5) | 16–18 | 28 | 14–16 | 35 | Compact cream-colored callus |

| Treatment | PGR Combination (mg/L) | Days to Callus Initiation (A. sapota) | Callogenesis % (A. sapota) | Days to Callus Initiation (T. cuneifolia) | Callogenesis % (T. cuneifolia) | Callus Characteristics |
|-----------|----------------------------------|---------------------------------------|----------------------------|---|--------------------------------|------------------------------------|
| T2 | NAA (1.0) + Kn (1.0) | 11–13 | 65 | 9–11 | 72 | Friable cream-white callus |
| T3 | NAA (1.0) + BAP (2.0) | 14–16 | 40 | 12–14 | 48 | Compact pale-yellow callus |
| T4 | 2,4-D (1.0) | 15–17 | 20 | 13–15 | 25 | Creash-colored callus |
| T5 | 2,4-D (1.0) + BAP (2.0) | 16–18 | 25 | 14–16 | 32 | Compact pale-colored callus |
| T6 | NAA (1.0) + Kn (1.0) + BAP (2.0) | 9–11 | 82* | 8–10 | 90* | Profuse friable cream-white callus |

*Significantly highest value ($p < 0.05$ vs. all other treatments; Tukey's test). Values represent mean of $n = 15$ explants per treatment.

3.2 Quantitative HPLC Analysis of Glycyrrhizin Content

Glycyrrhizin content in callus cultures varied significantly among PGR treatments and between the two plant species. *Taverniera cuneifolia* consistently accumulated higher levels of glycyrrhizin compared to *Achras sapota* under identical treatment conditions. Callus cultures maintained under T6 exhibited the maximum glycyrrhizin accumulation: 6.42 mg/g DW in *Achras sapota* and 9.84 mg/g DW in *Taverniera cuneifolia*. These values represented 3.13-fold and 2.64-fold increases, respectively, relative to glycyrrhizin

levels detected in field-grown plant material (Table 2). The 2,4-D-alone treatment (T4) was associated with the lowest in vitro glycyrrhizin accumulation in both species, consistent with its strong dedifferentiating effect, which may divert metabolic flux away from terpenoid biosynthesis [24].

Table 2. Glycyrrhizin Content in Callus Cultures Under Different PGR Combinations

| Treatment | Glycyrrhizin Content (mg/g DW) in <i>Achras sapota</i> | Glycyrrhizin Content (mg/g DW) in <i>Taverniera cuneifolia</i> |
|-------------------|--|--|
| Field-grown plant | 2.05 ± 0.12 | 3.72 ± 0.18 |
| T1 | 2.84 ± 0.09 | 4.65 ± 0.14 |
| T2 | 4.73 ± 0.21 | 7.11 ± 0.27 |
| T3 | 3.86 ± 0.17 | 6.24 ± 0.22 |
| T4 | 2.48 ± 0.11 | 4.12 ± 0.15 |
| T5 | 3.12 ± 0.14 | 5.33 ± 0.19 |
| T6 | 6.42 ± 0.31* | 9.84 ± 0.43* |

*Significantly highest value ($p < 0.05$). Values are mean ± SD ($n = 3$ independent experiments).

4. DISCUSSION

The results of the present study clearly demonstrate the pivotal role of PGR combination in modulating both callogenesis and secondary metabolite biosynthesis in callus cultures of *Achras sapota* and *Taverniera cuneifolia*. The superior performance of the ternary combination T6 [NAA (1.0 mg/L) + Kn (1.0 mg/L) + BAP (2.0 mg/L)] in both callus induction and glycyrrhizin accumulation is consistent with the established principle that simultaneous activation of multiple phytohormone signaling pathways produces synergistic effects on cellular proliferation and biosynthetic enzyme expression [9, 11].

The higher callogenesis frequency and glycyrrhizin content observed in *Taverniera cuneifolia* compared to *Achras sapota* corroborate earlier reports by Awad et al. [6], who demonstrated that *T. cuneifolia* possesses an innate biosynthetic capacity for glycyrrhizic acid equivalent to or exceeding that of *Glycyrrhiza glabra* in organized root cultures. The present study extends these observations to unorganized callus culture systems and, for the first time, establishes a quantitative comparison between *T. cuneifolia* and *A. sapota* under identical in vitro conditions.

The inverse relationship between 2,4-D concentration and glycyrrhizin accumulation (T4) observed in the present study aligns with reports indicating that high concentrations of 2,4-D induce profound somatic dedifferentiation and suppress secondary metabolite biosynthetic activity by downregulating transcript levels of key terpene synthase genes [24, 25]. In contrast, the balanced NAA:cytokinin ratio in T6 appears to maintain

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a state of partial differentiation conducive to secondary metabolite production a phenomenon previously described in other triterpenoid-accumulating callus systems [12, 22].

The 3.13-fold and 2.64-fold enhancements of glycyrrhizin in *Achras sapota* and *Taverniera cuneifolia* callus cultures, respectively, relative to field-grown plants under T6 are pharmaco-economically significant. Comparable or superior enhancements have been achieved through elicitor treatment, precursor feeding, and bioreactor-based culture systems in *Glycyrrhiza* species [3, 13, 25]. The friable, rapidly proliferating callus produced under T6 is an ideal starting material for establishing cell suspension cultures and, potentially, bioreactor scale-up, which could dramatically increase volumetric glycyrrhizin productivity [10, 23].

Taken together, the findings of the present study advance understanding of the hormonal regulation of glycyrrhizin biosynthesis in alternative plant hosts and provide a scientifically validated, optimized PGR protocol for sustained, scalable production of this high-value pharmaceutical secondary metabolite via plant tissue culture biotechnology.

5. CONCLUSION

The present study establishes that PGR optimization is a critical determinant of both callogenesis efficiency and glycyrrhizin accumulation in callus culture systems of *Achras sapota* and *Taverniera cuneifolia*. The ternary combination of NAA (1.0 mg/L) + Kinetin (1.0 mg/L) + BAP (2.0 mg/L) (T6) yielded the maximum callogenesis frequency (82% in *A. sapota*; 90% in *T. cuneifolia*) and the highest glycyrrhizin content (6.42 and 9.84 mg/g DW, respectively), representing over 2.6–3.1-fold improvement over field-grown material. *Taverniera cuneifolia* emerged as a superior alternative host for glycyrrhizin biosynthesis *in vitro*. The profuse friable callus generated provides an ideal platform for establishment of cell suspension cultures and bioreactor-based commercial production. Future studies should explore elicitor treatment, methyl jasmonate priming, and precursor feeding strategies to further enhance glycyrrhizin titers in these systems.

ABBREVIATIONS

BAP: 6-Benzylaminopurine; DW: Dry Weight; HPLC: High-Performance Liquid Chromatography; Kn: Kinetin; MS: Murashige and Skoog; NAA: α -Naphthaleneacetic Acid; PGR: Plant Growth Regulator; SD: Standard Deviation; 2,4-D: 2,4-Dichlorophenoxyacetic Acid

DECLARATIONS

Conflict of Interest: The authors declare no conflict of interest.

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Ethical Approval: Not applicable. The study involved plant material only.

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