Optimization of lipid compound synthesis in *Theobroma cacao* L. In vitro cultures through stress inducers: towards a sustainable approach

Optimización de la síntesis de compuestos lipídicos en cultivos in vitro de Theobroma cacao *L. mediante inductores de estrés: hacia un enfoque sostenible*

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Abstract

Theobroma cacao L., particularly the National "fino de aroma" variety, represents a strategic genetic resource for Ecuador; however, its cultivation is constrained by diseases and climate variability. This study aimed to characterize the fatty acid profile in biotechnological systems exposed to stress inducers (methyl jasmonate, salicylic acid, glycerol, and elevated temperature). Biomass proliferation was optimized in MS, B5, and WPM media, with WPM supplemented with NAA and KIN proving the most effective. Lipid extraction and quantification were conducted through acid hydrolysis, hexane extraction, and FTIR spectroscopy, focusing on oleic, palmitic, and stearic acids. Results indicated that salicylic acid and methyl jasmonate markedly enhanced fatty acid accumulation, whereas glycerol and high temperature had comparatively limited effects. These findings highlight the potential of stress inducers as effective tools to modulate lipid biosynthesis in cacao, offering promising applications for sustainable plant biotechnology.

Keywords: Fatty acids; FTIR spectroscopy; Plant biotechnology; *Theobroma cacao* L.

Resumen

Theobroma cacao L., particularmente la variedad Nacional "fino de aroma", representa un recurso genético estratégico para el Ecuador; sin embargo, su cultivo se ve limitado por enfermedades y la variabilidad climática. Este estudio tuvo como objetivo caracterizar el perfil de ácidos grasos en sistemas biotecnológicos expuestos a inductores de estrés (jasmonato de metilo, ácido salicílico, glicerol y temperatura elevada). La proliferación de biomasa se optimizó en los medios MS, B5 y WPM, siendo este último, suplementado con NAA y KIN, el más efectivo. La extracción y cuantificación de lípidos se realizaron mediante hidrólisis ácida, extracción con hexano y espectroscopía FTIR, con énfasis en los ácidos oleico, palmítico y esteárico. Los resultados indicaron que el ácido salicílico y el jasmonato de metilo incrementaron de manera notable la acumulación de ácidos grasos, mientras que el glicerol y la alta temperatura tuvieron efectos comparativamente más limitados. Estos hallazgos destacan el potencial de los inductores de estrés como herramientas eficaces para modular la biosíntesis de lípidos en cacao, ofreciendo aplicaciones prometedoras para una biotecnología vegetal sostenible.

Palabras clave: Ácidos grasos; Espectroscopía FTIR; Biotecnología vegetal; *Theobroma cacao* L.

Introduction

Cacao (*Theobroma cacao* L.), particularly the National "fino de aroma" ecotype, is a high-value genetic and economic resource for Ecuador (Mihai et al., 2022; Loor et al., 2019). Its distinctive sensory properties secure a premium position in the global market, yet cultivation faces threats from pathogens, climate change, and soil heavy metal accumulation (Ceccarelli et al., 2024; Jiménez et al., 2022; Oliva-Cruz et al., 2022). Cocoa butter, dominated by palmitic, stearic, and oleic acids, is crucial for chocolate production and has applications in pharmaceuticals, nutraceuticals, and cosmetics (Atanassova & Aayush, 2024; Loke et al., 2024). Despite its importance, lipid metabolism in *T. cacao* remains underexplored compared to other secondary metabolites, limiting the design of biotechnological strategies (Oracz et al., 2020).

In vitro culture systems enable precise investigation of metabolic regulation and stress inducers such as methyl jasmonate (MeJA), salicylic acid (SA), glycerol, and temperature shifts can modulate fatty acid biosynthesis, leading to distinct lipid profiles (Woch et al., 2023; Santos et al., 2024; Contreras et al., 2022). This study investigates the lipid responses of *T. cacao* L. biotechnological systems to various stress inducers, providing insights into sustainable strategies to enhance the production of the National "fino de aroma" cacao.

Materials and methods

This study employed an experimental approach and was conducted at the Research and Development Unit (UODIDE) of the Faculty of Food Science and Engineering and Biotechnology (FCIAB), Technical University of Ambato.

Plant material and culture establishment

Biotechnological systems (callus cultures) were initiated in Murashige & Skoog (MS), Woody Plant Medium (WPM), and Gamborg B5 media supplemented with various combinations of auxins and cytokinins to optimize biomass proliferation. The specific treatments included α-naphthaleneacetic acid (NAA, 1 or 2 mg/L) combined with kinetin (KIN, 0.5 mg/L), and 2,4-dichlorophenoxyacetic acid (2,4-D, 1 or 2 mg/L) combined with 6-benzylaminopurine (BAP, 0.5 mg/L), along with control treatments without growth regulators.

Application of stress inducers

After determining the optimal medium, homogeneous calli were transferred to stress conditions with four treatments: MeJA (150 µM), SA (50 µM), glycerol (3 %), and thermal stress (35 °C), alongside unstressed control. Each treatment was applied in three biological replicates and evaluated at 7, 14, 21, and 28 days.

Lipid extraction and analysis

Biomass was harvested, dried, and subjected to acid hydrolysis followed by automated hexane extraction. Fatty acid quantification was performed using Fourier-transform infrared (FTIR) spectroscopy, with stearic acid calibration curves serving as the standard.

Experimental design and statistical analysis

The experiment followed a factorial A×B design, with culture conditions and stress inducers as factors, to evaluate their effects on biomass accumulation and fatty acid production in *T. cacao* L. biotechnological systems. Data were analyzed using multifactor ANOVA in Minitab 18 to determine the main effects and interactions between factors, followed by Tukey's test for multiple comparisons ($p < 0.05$).

Results and discussion

Optimization of the culture medium was critical for biomass proliferation in *Theobroma cacao* L., with WPM supplemented with NAA (2 mg/L) and KIN (0.5 mg/L) proving most effective. Under these conditions, friable calli exhibiting higher metabolic activity were obtained (Gharat et al., 2025). Stress inducers exerted contrasting effects: glycerol and the unstressed control promoted greater biomass accumulation, whereas MeJA and SA reduced fresh callus weight but significantly enhanced fatty acid production, peaking during the third week (8.1 and 10.4 mg/mL, respectively). This pattern aligns with reports in plant and microalgal systems, where stress exposure activates metabolic pathways associated with lipid accumulation as an adaptive response (Ullah et al., 2023; Xu et al., 2017). Elevated temperature also induced a differential lipid profile, favoring the accumulation of saturated fatty acids, consistent with observations by Ahmadizadeh et al. (2020).

These results indicate that lipid biosynthesis in *T. cacao* can be modulated by specific stress inducers, providing a valuable biotechnological model to study cacao physiology under adverse conditions and to explore industrial applications for sustainable lipid metabolite production. The findings underscore the utility of in vitro cultures as experimental tools and highlight the need for further studies to precisely characterize fatty acid profiles and their regulation under diverse stress scenarios.

Conclusions

Optimization of the *Theobroma cacao* L. biotechnological system enabled the establishment of an efficient method for the proliferation of friable calli, with WPM medium supplemented with NAA (2 mg/L) and KIN (0.5 mg/L) proving most effective for biomass accumulation and metabolic activity.

Stress inducers modulated both biomass growth and fatty acid biosynthesis. MeJA and SA reduced initial proliferation but stimulated lipid accumulation, whereas glycerol and the control favored biomass growth. Lipid production peaked at 21 days, as confirmed by FTIR analysis of saturated and unsaturated fatty acids, demonstrating the system's capacity to generate metabolites of industrial interest.

These findings indicate that *T. cacao* L. calli, under optimal culture conditions and stress induction, represent a viable biotechnological system for fatty acid production and potential bioactive metabolites. Future research should integrate complementary techniques such as GC-MS for more precise lipid characterization, expand the range of fatty acids analyzed, and evaluate other relevant secondary metabolites to comprehensively understand metabolic responses to abiotic stress, thereby enhancing the biotechnological potential of the system.

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