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Evaluation of Soluble Sugar Content, Phenolic, and Amino-Acid Profiles of Three Cocoa (*Theobroma cacao* L.) Genotypes for Embryogenic Development

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Abstract

Somatic embryogenesis (SE) in cocoa (*Theobroma cacao* L.) offers significant potential for mass propagation of elite genotypes, but the metabolic changes needed for successful regeneration are not well understood. This study investigated temporal changes in amino acid, phenolic, and soluble sugar profiles during callus development in three cocoa genotypes (TC-1/G5, TC-8/G12, and T86/2/G1) cultured on hormone-supplemented media. Staminode explants were induced on 1 mg/L 2,4-D and 0.2 mg/L TDZ, then developed on 1 mg/L 2,4-D and 1 mg/L 6-BA. Metabolite analyses were conducted at 4, 6, and 8 weeks of culture. Results revealed highly significant genotype $\times$ week interactions for all metabolites ($P < 0.01$), indicating distinct metabolic strategies among genotypes. Correlation analysis identified three co-regulated metabolic modules, a serine-centered network linking one-carbon metabolism and stress responses, an aspartate-glutamine axis governing nitrogen assimilation, and lysine functioning as a negative feedback regulator. Temporal flux analysis revealed a three-phase metabolic model: metabolic priming (Week 4), quiescence and reset (Week 6), and coordinated pathway reactivation (Week 8). The C/N balance ratio showed genotype-specific patterns, with G5 exhibiting dramatic shifts potentially associated with organogenic transitions. These findings provide mechanistic insights into the effect of genetic factors and physiological developments of the callus on

embryogenic capacity of floral explants in *T. cacao*, showing coordinated pathway reactivation supporting regeneration competence.

Keywords: In-vitro culture, Metabolites, Cocoa, Nutrients mobilization, Somatic embryogenesis.

Introduction

Cocoa (*Theobroma cacao* L.) is a perennial tropical crop of immense economic importance, serving as the primary source of raw material for the global chocolate industry valued at over \$130 billion annually (Kongor *et al.*, 2024; Patel *et al.*, 2023). West Africa, particularly Côte d'Ivoire and Ghana, accounts for approximately 70% of global cocoa production, with Nigeria contributing significantly to regional output (Oluyole *et al.*, 2022). However, the industry faces unprecedented challenges including aging plantations, climate variability, pest and disease pressure, and declining productivity, which collectively threaten supply stability and smallholder livelihoods (Läderach *et al.*, 2023; Tetteh *et al.*, 2024).

Conventional cocoa propagation through grafting and rooted cuttings, while effective for maintaining genetic fidelity, cannot meet the large-scale demand for high-quality planting material required to replace unproductive stands (Maximova *et al.*, 2021). Moreover, these methods are labor-intensive, season-dependent, and constrained by the availability of suitable rootstock and scion material (Guiltinan *et al.*, 2022). In-vitro propagation techniques, particularly somatic embryogenesis (SE), offer a transformative solution by enabling rapid, year round multiplication of elite genotypes with

superior yield, disease resistance, and quality traits (Ali *et al.*, 2023; Fister *et al.*, 2024).

Somatic embryogenesis in cocoa (*Theobroma cacao*) is a vital in vitro technique for mass-producing genetically identical, disease-free, and high-yielding clones, primarily using floral parts like staminodes and petals. It involves callus induction and embryo development, heavily dependent on genotype, media composition and hormones (2,4-D, TDZ). However, SE efficiency in cocoa remains highly genotype-dependent, with success rates varying from 0% to over 80% across different cultivars (Quiroz-Figueroa *et al.*, 2020; Tan and Furtek, 2023). This recalcitrance is attributed to complex interactions among endogenous hormone balances, oxidative stress, phenolic accumulation, and inadequate understanding of the metabolic transitions required for embryogenic commitment (García *et al.*, 2023). In vitro cultures are often heterotrophic or mixotrophic due to limited photosynthesis, and therefore rely heavily on the mobilization, uptake, and utilization of exogenous amino acids, soluble sugars, and phenolics as critical drivers of growth, differentiation, and metabolic health (Ferreira *et al.*, 2009; Geresua, 2024).

Recent metabolomic studies have started to reveal the biochemical reasons why cocoa

shows resistance to somatic embryogenesis (SE). Beguinod *et al.* (2024) demonstrated that the metabolome of *T. cacao* callus undergoes profound compositional shifts during somatic embryogenesis, with nitrogen-containing compounds and secondary metabolites exhibiting stage-specific accumulation patterns that differ across developmental transitions. Similarly, García *et al.* (2023) reported genotype-specific biochemical signatures in recalcitrant versus responsive cocoa genotypes, identifying differential regulation of phenolic metabolism and amino acid pools as key determinants of embryogenic competence. However, these studies have largely focused on single genotypes or broad metabolite categories under non Nigerian germplasm conditions, and the simultaneous temporal profiling of amino acid, phenolic, and soluble sugar dynamics across multiple genotypes at defined callus developmental stages remains poorly characterised. In particular, the coordinated interplay among these three metabolite classes and their genotype-specific trajectories during the critical callus to embryo transition has not been systematically investigated. The present study therefore aimed to analyse the amino acid, phenolic, and soluble sugar contents in cocoa callus over time to understand the metabolic pathways and strategies that support regeneration in different Nigerian genotypes.

Materials and Methods

Plant Material and Culture Establishment

Three cocoa genotypes, TC-1 (designated G5), TC-8 (designated G12), and T86/2 (designated G1) maintained in the germplasm collection at the Cocoa Research Institute of Nigeria (CRIN), Ibadan, were selected based on their contrasting agronomic performance and previously documented regeneration responses. Fresh, unopened flower buds were harvested from mature, healthy trees during the peak flowering period and transported to the laboratory in sterile containers on ice within 2 hours of collection.

Surface Sterilization and Explant Preparation

Flower buds were surface-sterilized following a modified protocol of PennState (2010). Buds were first rinsed under running tap water for 10 minutes, then immersed in 70% (v/v) ethanol for 1 minute, followed by treatment with 2.5% (w/v) sodium hypochlorite solution (commercial bleach) containing two drops of Tween-20 per 100 mL for 15 minutes under gentle agitation. The plant materials were rinsed three times with sterile distilled water. (5 minutes each), flower buds were dissected under aseptic conditions in a laminar flow hood. Staminode explants (approximately 3-5 mm in length) were carefully excised using sterile scalpels and forceps, avoiding damage to meristematic tissues.

Culture Media and Hormonal Treatments

Basal medium consisted of Driver and Kuniyuki Walnut (DKW) medium (Driver and Kuniyuki, 1984) supplemented with 20 g/L sucrose, 0.1 g/L myo-inositol, and solidified with

2 g/L Phytigel. The pH was adjusted to 5.8 ± 0.1 using 0.1 N NaOH or HCl before autoclaving at 121°C for 20 minutes.

For callus induction, staminode explants were cultured on DKW medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/L thidiazuron (TDZ). Following callus initiation (typically after 2 weeks), calli were subcultured onto development medium containing 1 mg/L 2,4-D and 1 mg/L 6-benzylaminopurine (6-BA). All plant growth regulators were filter-sterilized (0.22 µm membrane) and added to autoclaved medium cooled to approximately 55°C. Cultures were maintained at $25 \pm 2^\circ\text{C}$ under a dark condition.

Experimental Design and Sample Collection

The experiment was arranged in a factorial design with two factors: genotype (three levels: G1, G5, G12) and developmental week (three levels: 4, 6, and 8 weeks post-subculture onto development medium). Each treatment combination consisted of six biological replicates, with each replicate comprising pooled callus tissue from five culture vessels to minimize within treatment heterogeneity arising from positional variation within the culture cabinet and minor vessel to vessel fluctuations in microenvironment, thereby providing a more representative and reproducible measure of the metabolic state of each genotype-week treatment combination. At the designated time points (4, 6, and 8 weeks), actively growing calli exhibiting friable texture and pale yellow to cream coloration were harvested, immediately frozen in liquid nitrogen, and stored at -80°C until metabolite extraction.

Amino Acid Profiling

Amino acid analysis was conducted using the precolumn derivatization method with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) followed by reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection, as described by Cohen and Michaud (1993) with modifications. Briefly, 100 mg of frozen callus tissue was homogenized in 1 mL of 0.1 N HCl using a pre-chilled mortar and pestle. Homogenates were centrifuged at $15,000 \times g$ for 20 minutes at 4°C, and supernatants were filtered through 0.22 µm PVDF syringe filters. Filtered extracts (10 µL) were derivatized with AQC reagent according to the manufacturer's protocol (Waters AccQ•Tag Chemistry Package).

Chromatographic separation was performed on a Waters Alliance 2695 HPLC system equipped with a Waters 2475 Multi-λ Fluorescence Detector (excitation: 250 nm; emission: 395 nm). An AccQ•Tag amino acid column (Nova-Pak C18, 4 µm, 150×3.9 mm; Waters Corporation) thermostated at 37°C was employed. Mobile phase gradients consisted of Eluent A (AccQ•Tag Eluent A Concentrate diluted 10-fold with Milli-Q water), Eluent B (100% acetonitrile, HPLC grade), and Eluent C (Milli-Q water), delivered according to the standard Waters AccQ•Tag gradient profile at a flow rate of 1.0 mL/min. Amino acids were quantified by comparison with external standards (Amino Acid Standard H, Thermo Scientific Pierce) ranging from 2.5 to 250 pmol, and results were expressed as µmol/g fresh weight (FW).

Total Phenolic Content Determination

Total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu (FC) assay as described by Magalhães *et al.* (2010). Frozen callus samples (200 mg) were extracted with 2 mL of 80% (v/v) aqueous methanol by vortexing for 2 minutes followed by sonication for 15 minutes at room temperature. After centrifugation ($12,000 \times g$, 15 minutes, 4°C), 100 μL of supernatant was mixed with 500 μL of 10-fold diluted FC reagent and allowed to stand for 5 minutes. Subsequently, 400 μL of 7.5% (w/v) sodium carbonate solution was added, and the mixture was incubated at room temperature in the dark for 60 minutes. Absorbance was measured at 765 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific) against a reagent blank. Gallic acid was used as the reference standard (concentration range: 10-100 $\mu\text{g/mL}$), and total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram FW.

Total Soluble Sugar Quantification

Total soluble sugar content was estimated using the phenol-sulfuric acid colorimetric method described by Onitilo *et al.* (2007) with minor modifications. Callus samples (20 mg) were homogenized in 1 mL of 80% (v/v) ethanol and subsequently extracted with 10 mL of hot 80% ethanol at 80°C for 15 minutes. Following addition of 2 mL of distilled water, mixtures were vortexed vigorously and centrifuged at $3,000 \times g$ for 10 minutes. To 200 μL of supernatant, 200 μL of 5% (w/v) phenol solution and 1 mL of concentrated sulfuric acid (95-97%) were added sequentially. After incubation at room temperature for 30 minutes to allow color development, absorbance was measured at 490 nm using the Genesys 10S spectrophotometer. D-glucose was employed as the standard (concentration range: 10-100 $\mu\text{g/mL}$), and total soluble sugar content was expressed as milligrams of glucose equivalents per gram FW.

Statistical Analysis

Metabolite measurements (amino acids, total phenolics, soluble sugars) were analyzed from three genotypes across callus development weeks, with $n = 6$ biological replicates per treatment combination. Prior to analysis, model residuals were evaluated for normality using the Shapiro-Wilk test, and homogeneity of variance among genotypes was assessed using Levene's test (Table S1). Analysis of variance (ANOVA) was conducted using the SAS 9.4 GLM procedure with genotype, week, and genotype $\times$ week included as fixed effects. Given the balanced factorial design and equal replication across treatments, ANOVA was considered robust to moderate deviations from normality. When significant effects were detected ($P < 0.05$), treatment means were separated using Fisher's Least Significant Difference (LSD) test at $\alpha = 0.05$.

Pearson correlations identified co-regulated metabolites. Multivariate analyses included PCA (biplot visualization) and hierarchical clustering (heatmaps) performed in R 4.3.1 using stats,

ggplot2 (3.4.2), ggbiplot, pheatmap (1.0.12), and corplot (0.92) packages. Clustering used Euclidean distance and Ward's linkage.

Results

Assumption Testing

Prior to ANOVA, model residuals were evaluated for normality using the Shapiro-Wilk test, and homogeneity of variance among genotypes was assessed using Levene's test (Table S1). Shapiro-Wilk tests indicated that 10 of the 19 metabolite variables followed a normal distribution ($P > 0.05$), while several amino acids showed deviations from normality. Levene's test indicated homogeneous variances for most variables (17 of 19), with only threonine and total soluble sugar exhibiting significant heterogeneity among genotypes ($P < 0.05$). Given the balanced factorial design and equal replication across treatments, ANOVA was considered robust to these moderate deviations.

Effects of Genotype, Developmental Week, and Their Interaction on Metabolite Profiles

Analysis of variance revealed highly significant effects ($P < 0.01$) of genotype, developmental week, and their interaction on all amino acid profiles in cocoa callus (Table 1). Genotype effects were particularly pronounced for proline (mean square [MS] = 17.58), methionine (MS = 7.02), and histidine (MS = 2.32), indicating strong genetic variation in nitrogen metabolism among G1, G5, and G12. Week effects showed the largest mean squares for cysteine (MS = 18.87), methionine (MS = 7.41), and proline (MS = 14.18), demonstrating that these stress-responsive amino acids undergo dramatic temporal changes during callus development. The significant genotype $\times$ week interactions for all amino acids, with particularly high values for glutamine (MS = 10.49), cysteine (MS = 16.19), and alanine (MS = 8.79), confirm that genotypes respond differently to developmental cues, suggesting distinct metabolic strategies for regeneration competence.

For primary and secondary metabolites, both total phenolics and total sugars showed significant genotypic differences (Table 2). While sugar levels remained relatively stable across developmental weeks (non-significant week effect), phenolic content varied significantly with time, and both metabolites exhibited significant genotype $\times$ week interactions, indicating coordinated but genotype-specific regulation of carbon allocation between primary and secondary metabolism during callus maturation.

Mean metabolite concentrations ($\pm$ SE) across all genotype-week treatment combinations are presented in Table 3. Amino acid concentrations varied considerably across genotypes and developmental stages, with proline and cysteine recording the highest values overall, particularly in G12 at Week 4 (6.83 ± 0.01 and 7.75 ± 0.03 $\mu\text{mol/g FW}$, respectively) and G5 at Week 4 (5.625 ± 0.025 and 7.455 ± 0.015 $\mu\text{mol/g FW}$, respectively), consistent with the high mean squares observed for these amino acids in Table 1. A general trend of declining amino acid concentrations from Week 4 to Week 6, followed by partial recovery at Week 8, was evident across genotypes, supporting the three-phase metabolic model described. Total

phenolic content was highest in G5 at Week 4 (0.385 ± 0.005 mg GAE/g FW) and generally increased toward Week 8, while total soluble sugar content showed genotype-specific patterns, with G12 recording the highest sugar concentration at Week 4 (0.555 ± 0.005 mg glucose eq./g FW).

Table 1: Mean squares of three *cacao* genotypes at three different weeks with respect to Amino acids profiles from the callus

Source	Df	ASP	SER	GLU	GLY	HIS	THR	ARG	ALA	PRO	CYS	TYR	VAL	MET	LYS	ISO_L	LEU	PHE
Genotype	2	0.13**	1.99**	4.99**	0.72**	2.32**	0.07**	1.17**	3.59**	17.58*	2.09**	0.62**	0.04**	7.02**	0.17**	0.19**	0.16**	1.21**
Week	2	0.11**	3.33**	1.94**	5.31**	5.85**	0.24**	2.02**	1.19**	14.18*	18.87*	0.41**	0.04**	7.41**	0.11**	0.32**	1.05**	2.69**
Genotype *Week	4	0.10**	0.99**	10.49*	5.15**	1.05**	0.69**	1.14**	8.79**	6.94**	16.19*	0.34**	0.11**	2.93**	0.06**	0.14**	1.06**	1.28**
Error	9	0.0003	0.05	0.001	0.001	0.01	0.001	0.0003	0.02	0.01	0.03	0.0001	0.001	0.001	0.001	0.001	0.06	0.01

** indicates significance at $P < 0.01$. Df = degrees of freedom

Table 2: Mean squares of Total Phenols and Sugar profiles from the callus of three *cacao* genotypes at three different weeks

Source	Df	Total Phenols	Total Sugar
Genotype	2	0.01**	0.01**
Week	2	0.003**	0.01 ^{ns}
Genotype x Week	4	0.001**	0.02**
Error	9	0.0002	0.002

** indicates statistical significance at $P < 0.01$, while ns denotes non-significant effects. Df = degrees of freedom

Table 3: Mean ($\pm$ SE) Metabolite Concentrations in Cocoa Callus Across Genotypes and Culture Weeks

Genotype	Week	Total Sugar	Total Phenolics	ASP	SER	GLU	GLY	HIS	THR	ARG	ALA	PRO	CYS	TYR	VAL	MET	LYS	ILE	LEU	PHE
G1	4	0.465 $\pm$ 0.015	0.28 $\pm$ 0.01	0.725 $\pm$ 0.015	2.395 $\pm$ 0.005	2.305 $\pm$ 0.015	2.77 $\pm$ 0	2.32 $\pm$ 0.01	0.73 $\pm$ 0.01	1.975 $\pm$ 0.005	4.38 $\pm$ 0	5.655 $\pm$ 0.205	7.035 $\pm$ 0.015	1.215 $\pm$ 0.005	0.13 $\pm$ 0	3.53 $\pm$ 0.02	0.16 $\pm$ 0.01	0.845 $\pm$ 0.035	1.665 $\pm$ 0.005	2.295 $\pm$ 0.065
	6	0.435 $\pm$ 0.005	0.3 $\pm$ 0.01	0.705 $\pm$ 0.015	0.2 $\pm$ 0.01	0.775 $\pm$ 0.015	0.045 $\pm$ 0.015	0.04 $\pm$ 0	0.05 $\pm$ 0	0.05 $\pm$ 0	0.45 $\pm$ 0.01	0.3 $\pm$ 0.01	0.125 $\pm$ 0.005	0.115 $\pm$ 0.005	0.17 $\pm$ 0	0.015 $\pm$ 0.005	0.61 $\pm$ 0.01	0.155 $\pm$ 0.005	0.145 $\pm$ 0.005	0.125 $\pm$ 0.005
	8	0.415 $\pm$ 0.035	0.335 $\pm$ 0.005	1.4 $\pm$ 0.01	0.63 $\pm$ 0.49	6.645 $\pm$ 0.015	0.66 $\pm$ 0.02	0.045 $\pm$ 0.015	1.69 $\pm$ 0.02	1 $\pm$ 0.02	0.445 $\pm$ 0.015	1.13 $\pm$ 0.02	8.03 $\pm$ 0.36	0.83 $\pm$ 0	0.175 $\pm$ 0.015	0.015 $\pm$ 0.005	0.62 $\pm$ 0	0.15 $\pm$ 0.01	1.58 $\pm$ 0.53	1.05 $\pm$ 0.01
G12	4	0.555 $\pm$ 0.005	0.32 $\pm$ 0.01	0.69 $\pm$ 0.01	3.03 $\pm$ 0.02	2.96 $\pm$ 0.03	0.175 $\pm$ 0.005	2.575 $\pm$ 0.025	0.865 $\pm$ 0.005	2.7 $\pm$ 0.01	0.635 $\pm$ 0.025	6.83 $\pm$ 0.01	7.75 $\pm$ 0.03	0.1 $\pm$ 0.01	0.58 $\pm$ 0.01	3.965 $\pm$ 0.015	0.015 $\pm$ 0.005	0.945 $\pm$ 0.015	2.025 $\pm$ 0.045	2.8 $\pm$ 0.21
	6	0.495 $\pm$ 0.045	0.255 $\pm$ 0.005	0.665 $\pm$ 0.005	1.595 $\pm$ 0.005	2.485 $\pm$ 0.055	2.695 $\pm$ 0.065	1.47 $\pm$ 0.24	0.49 $\pm$ 0.02	1.28 $\pm$ 0.01	4.325 $\pm$ 0.325	4.3 $\pm$ 0.03	4.495 $\pm$ 0.005	0.11 $\pm$ 0.01	0.145 $\pm$ 0.005	2.195 $\pm$ 0.025	0.285 $\pm$ 0.015	0.495 $\pm$ 0.045	0.94 $\pm$ 0.01	1.235 $\pm$ 0.005
	8	0.55 $\pm$ 0	0.34 $\pm$ 0.02	0.7 $\pm$ 0.01	1.76 $\pm$ 0.01	1.935 $\pm$ 0.005	0.05 $\pm$ 0	0.02 $\pm$ 0	0.54 $\pm$ 0.01	1.47 $\pm$ 0.01	1.21 $\pm$ 0.01	4.695 $\pm$ 0.005	6.175 $\pm$ 0.005	0.935 $\pm$ 0.005	0.16 $\pm$ 0.01	2.975 $\pm$ 0.055	0.095 $\pm$ 0.005	0.64 $\pm$ 0.01	1.315 $\pm$ 0.005	1.76 $\pm$ 0.01
G5	4	0.325 $\pm$ 0.005	0.385 $\pm$ 0.005	0.705 $\pm$ 0.005	2.3 $\pm$ 0.04	0.74 $\pm$ 0.01	1.025 $\pm$ 0.005	2.27 $\pm$ 0.07	0.745 $\pm$ 0.025	1.865 $\pm$ 0.005	0.255 $\pm$ 0.005	5.625 $\pm$ 0.025	7.455 $\pm$ 0.015	0.085 $\pm$ 0.005	0.15 $\pm$ 0	3.525 $\pm$ 0.025	0.225 $\pm$ 0.005	0.725 $\pm$ 0.025	1.695 $\pm$ 0.005	2.25 $\pm$ 0.05
	6	0.6 $\pm$ 0.05	0.365 $\pm$ 0.005	0.68 $\pm$ 0.02	2.645 $\pm$ 0.005	3.025 $\pm$ 0.015	3.755 $\pm$ 0.015	2.68 $\pm$ 0.01	0.885 $\pm$ 0.035	2.43 $\pm$ 0.01	0.305 $\pm$ 0.015	7.21 $\pm$ 0.01	7.025 $\pm$ 0.015	0.085 $\pm$ 0.005	0.605 $\pm$ 0.045	4.1 $\pm$ 0.02	0.135 $\pm$ 0.025	0.96 $\pm$ 0.03	1.95 $\pm$ 0.05	2.64 $\pm$ 0.04
	8	0.51 $\pm$ 0.05	0.38 $\pm$ 0	0.69 $\pm$ 0.01	1.065 $\pm$ 0.025	0.51 $\pm$ 0.03	0.15 $\pm$ 0.01	1.18 $\pm$ 0.02	0.315 $\pm$ 0.015	0.87 $\pm$ 0.02	1.22 $\pm$ 0.02	3.3 $\pm$ 0.02	3.605 $\pm$ 0.035	0.075 $\pm$ 0.005	0.14 $\pm$ 0.01	1.59 $\pm$ 0.03	0.42 $\pm$ 0.03	0.365 $\pm$ 0.015	0.555 $\pm$ 0.005	0.92 $\pm$ 0

Relationships Among Metabolites During Callus Regeneration

Pearson correlation analysis revealed three major co-regulated metabolic modules (Table 4, Fig. 1). The serine-centered module showed exceptionally strong positive correlations ($r > 0.94$) among serine, arginine, proline, methionine, isoleucine, and phenylalanine, indicating coordinated regulation of one-carbon metabolism, transamination pathways, and aromatic amino acid biosynthesis. This metabolic network likely supports the synthesis of S-adenosylmethionine (SAM) through methionine, which functions as a universal methyl-group donor and a precursor for polyamine biosynthesis (via arginine), while proline contributes to osmotic adjustment and stress protection, processes that are essential for sustained cell division and successful regeneration.

Lysine exhibited strong negative correlations with this serine-centered network ($r = -0.87$ to -0.95 with arginine, proline, methionine, and isoleucine), suggesting it functions as a negative feedback regulator, possibly through inhibition of aspartate kinase in the aspartate family biosynthetic pathway. The aspartate-glutamine-threonine module ($r = 0.84$ - 0.90) represents second coordinated network linking nitrogen assimilation (glutamine) with aspartate family metabolism, critical for supplying carbon skeletons and nitrogen for amino acid biosynthesis. Cysteine showed significant positive correlations with leucine ($r = 0.89$), threonine ($r = 0.80$), and aromatic amino acids ($r = 0.79$ with phenylalanine), indicating glutathione biosynthesis is coupled to overall transamination flux and redox buffering capacity scales with amino acid biosynthetic activity. The correlation heatmap (Fig. 1) visually confirms these three distinct metabolic modules: the serine-centered network (red cluster), the aspartate-glutamine axis (orange cluster), and lysine as an isolated negative regulator (blue).

Table 4: Correlation coefficients among the amino acids profiles from the *cacao* genotypes tested

	ASP	SER	GLU	GLY	HIS	THR	ARG	ALA	PRO	CYS	TYR	VAL	MET	LYS	ISO_L	LEU	PHE
ASP	1																
SER	-0.43 ^{ns}	1															
GLU	0.84**	-0.05 ^{ns}	1														
GLY	-0.17 ^{ns}	0.43 ^{ns}	0.16 ^{ns}	1													
HIS	-0.45 ^{ns}	0.85**	-0.15 ^{ns}	0.58*	1.00												
THR	0.80**	0.17 ^{ns}	0.90**	0.16 ^{ns}	0.08 ^{ns}	1.00											
ARG	-0.24 ^{ns}	0.96**	0.16 ^{ns}	0.44 ^{ns}	0.82**	0.38 ^{ns}	1.00										
ALA	-0.22 ^{ns}	0.12 ^{ns}	-0.05 ^{ns}	0.48*	0.16 ^{ns}	-0.18 ^{ns}	0.04 ^{ns}	1.00									
PRO	-0.51*	0.96**	-0.12 ^{ns}	0.50*	0.85**	0.10 ^{ns}	0.94**	0.14 ^{ns}	1.00								
CYS	0.34 ^{ns}	0.65**	0.56*	0.27 ^{ns}	0.47*	0.80**	0.79**	-0.04 ^{ns}	0.61**	1.00							
TYR	0.40 ^{ns}	-0.02 ^{ns}	0.39 ^{ns}	0.04 ^{ns}	-0.28 ^{ns}	0.36 ^{ns}	0.02 ^{ns}	0.38 ^{ns}	-0.08 ^{ns}	0.36 ^{ns}	1.00						
VAL	-0.18 ^{ns}	0.60**	0.22 ^{ns}	0.27 ^{ns}	0.56*	0.24 ^{ns}	0.68**	-0.38 ^{ns}	0.59**	0.34 ^{ns}	-0.37 ^{ns}	1.00					
MET	-0.57*	0.96**	-0.21 ^{ns}	0.45*	0.82**	0.02 ^{ns}	0.91**	0.14 ^{ns}	0.99**	0.57*	-0.03 ^{ns}	0.52*	1.00				
LYS	0.56*	-0.92**	0.18 ^{ns}	-0.31 ^{ns}	-0.67**	0.02 ^{ns}	-0.87**	-0.18 ^{ns}	-0.94**	-0.53*	-0.06 ^{ns}	-0.49*	-0.95**	1.00			
ISO_L	-0.52*	0.96**	-0.12 ^{ns}	0.49*	0.84**	0.08 ^{ns}	0.94**	0.13 ^{ns}	0.97**	0.58*	0.01 ^{ns}	0.62**	0.98**	-0.93**	1.00		
LEU	0.14 ^{ns}	0.82**	0.45 ^{ns}	0.37 ^{ns}	0.60**	0.67**	0.87**	-0.08 ^{ns}	0.72**	0.89**	0.23 ^{ns}	0.56*	0.69**	-0.64**	0.73**	1.00	
PHE	-0.25 ^{ns}	0.96**	0.11 ^{ns}	0.42 ^{ns}	0.80**	0.35 ^{ns}	0.99**	0.01 ^{ns}	0.94**	0.79**	0.08 ^{ns}	0.62**	0.93**	-0.87**	0.95**	0.87**	1.00

** = significant at 5% level of probability, ns = non-significant, aspartic acid (Asp), serine (Ser), glutamic acid (Glu), glycine (Gly), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), cysteine (Cys), tyrosine (Tyr), valine (Val), methionine (Met), lysine (Lys), isoleucine (Ile), leucine (Leu), and phenylalanine (Phe)

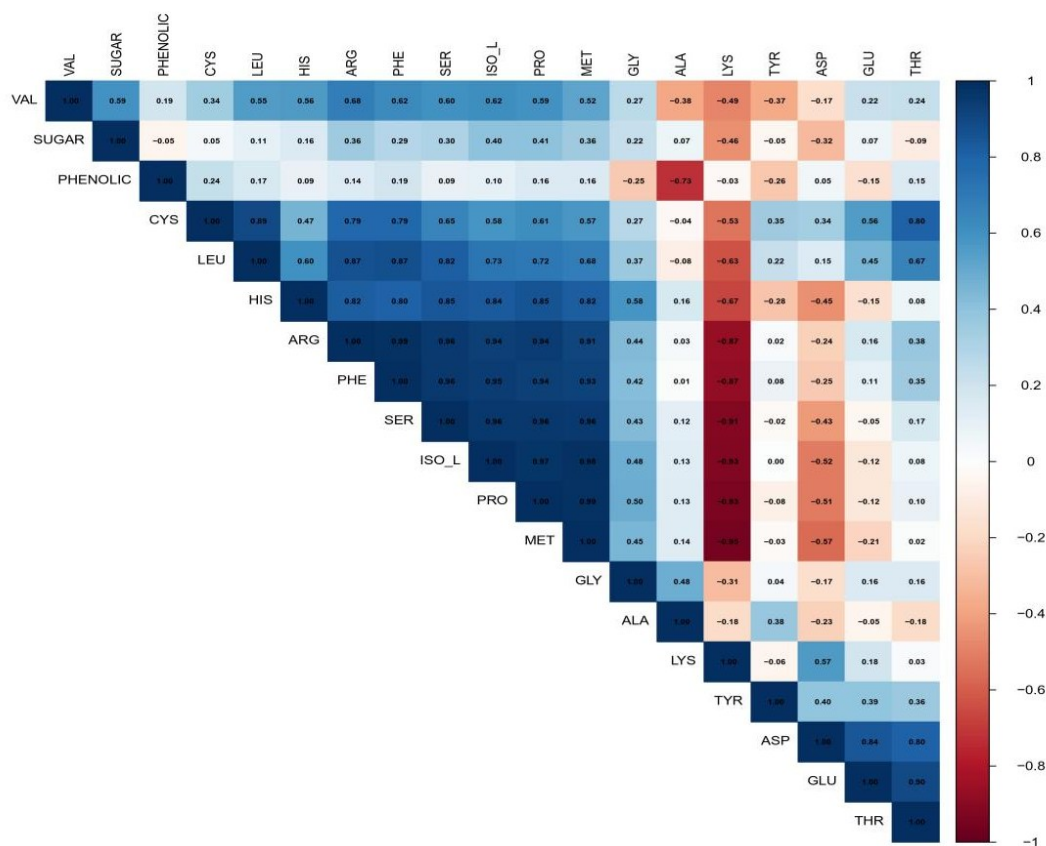


Figure 1. Correlation matrix heatmap showing relationships among amino acid metabolites in cocoa callus cultures.

Strong positive correlations are indicated in red, strong negative correlations in blue, and weak correlations in white. Three major metabolic modules are evident: the serine-centered stress/growth network, the aspartate-glutamine nitrogen assimilation axis, and lysine as a negative feedback regulator.

Metabolite Loading Patterns and Hierarchical Clustering Analysis

Variable loading analysis through PCA biplot visualization (Fig. 2) identified distinct metabolite clusters corresponding to coordinated biosynthetic pathways and regulatory networks. PC1 loading vectors demonstrated the serine-proline-aromatic amino acid stress/growth module, with serine (0.987), arginine (0.988), proline (0.978), methionine (0.957), isoleucine (0.976), and phenylalanine (0.984) showing strong positive correlations, while lysine (-0.905) exhibited opposing regulation. PC2 loading patterns revealed the aspartate-threonine-glutamine nitrogen assimilation module, with aspartate (0.928), glutamine (0.916), and threonine (0.955) forming a distinct functional cluster. Hierarchical clustering analysis (Fig. 3) confirmed these multivariate patterns, grouping metabolites into functional clusters corresponding to biosynthetic pathways. The dendrogram reveals three major metabolite clusters: (1) the serine-proline-aromatic amino acid stress/growth module, (2) the

aspartate-threonine-glutamine nitrogen assimilation module, and (3) branched-chain amino acids with leucine. Sample clustering shows clear separation by developmental week, with Week 4 and Week 8 forming distinct groups while Week 6 samples show intermediate metabolic states, supporting the hypothesis of a metabolic reset phase at this critical developmental checkpoint.

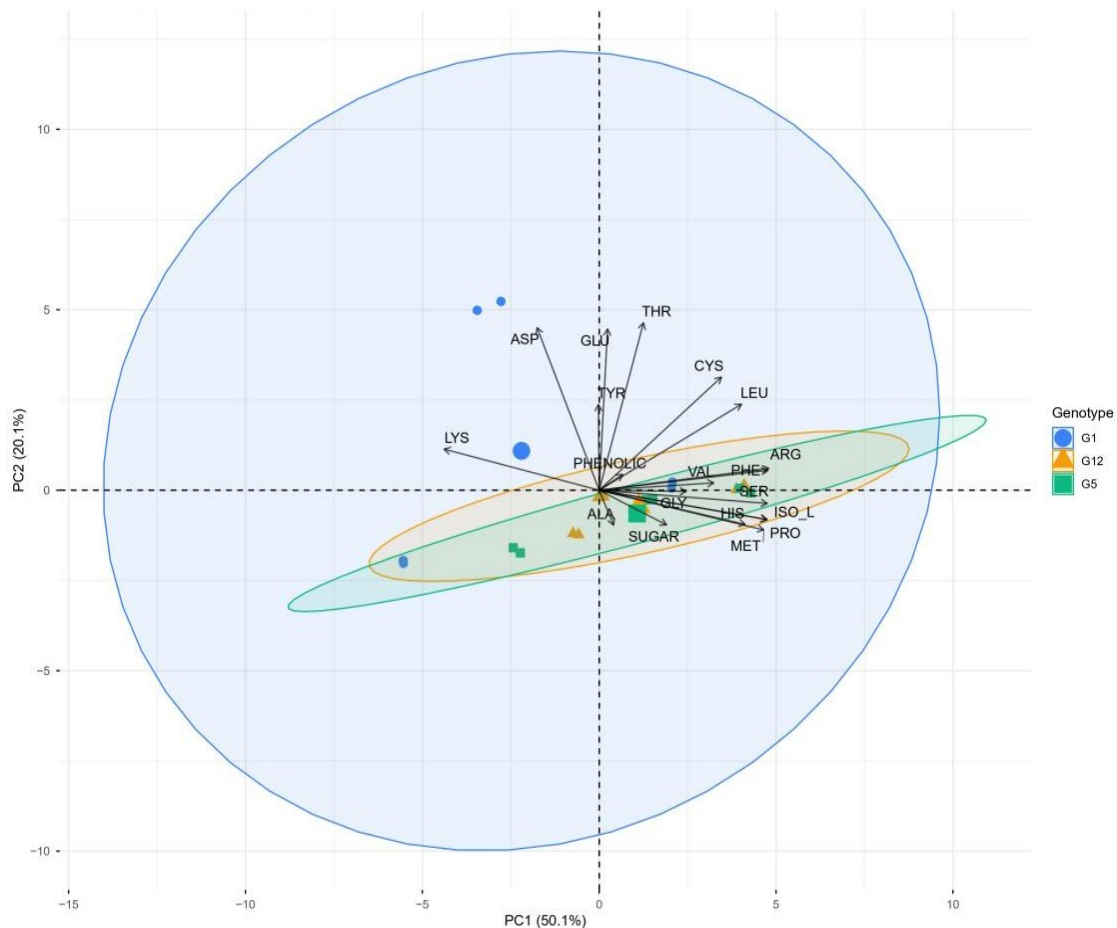


Figure 2. Principal component analysis (PCA) biplot showing variable loadings for amino acid metabolites and sample distribution across developmental weeks.

PC1 and PC2 collectively explain 82.0% of total variance. Arrows indicate the direction and magnitude of variable contributions, with longer arrows representing stronger influences on sample separation.

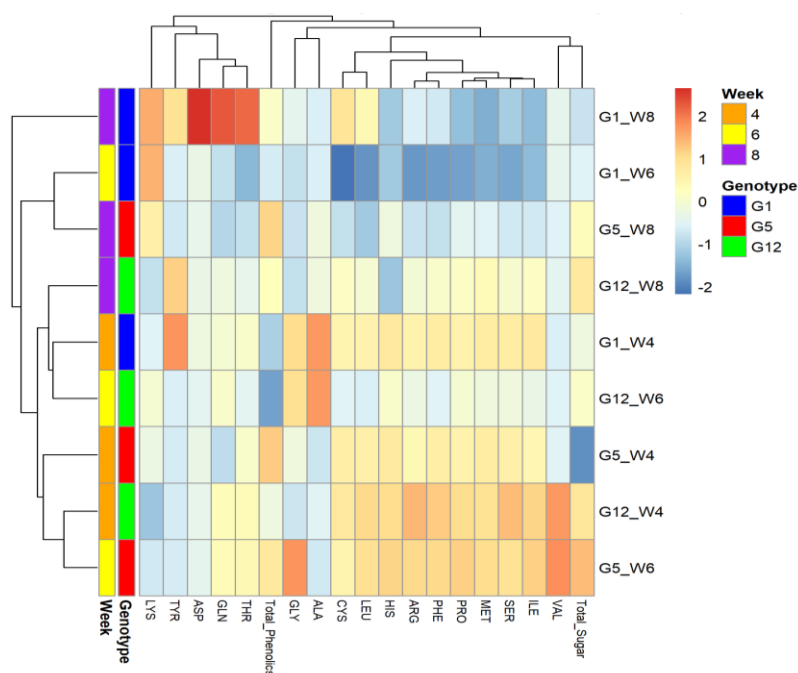


Figure 3. Hierarchical clustering heatmap of amino acid metabolites across genotypes and developmental weeks.

Color intensity represents scaled metabolite concentrations (red = high, blue = low). Clear separation of developmental weeks indicates distinct metabolic phases during callus maturation.

Biosynthetic Flux Dynamics During Callus Regeneration

Temporal analysis of pathway flux ratios revealed distinct metabolic transitions underlying regeneration competence (Fig. 4). The C/N balance ratio (sugar/amino acids) remained relatively stable across development but showed genotype-specific patterns, with G5 exhibiting a dramatic increase from Week 4 to Week 8, suggesting a shift from nitrogen-rich to more balanced carbon-nitrogen metabolism potentially associated with organogenic transitions.

The stress/growth metabolite ratio (Pro+Cys/Gln+Arg) showed pronounced temporal dynamics. G1 decreased from high stress at Week 4 (ratio ~3.0) to metabolic minimum at Week 6 (ratio ~0.5), then recovered to balanced state at Week 8 (ratio ~1.2), supporting the metabolic reset hypothesis. G5 maintained consistently elevated stress ratios (>2.5) throughout development, indicating a constitutive stress-priming strategy. G12 showed intermediate patterns with gradual stress ratio elevation from Week 4 to Week 8, suggesting accumulation of protective metabolites during maturation.

Shikimate pathway flux, indicated by the phenylalanine/tyrosine ratio, exhibited extreme values at Week 4 for G12 and G5 (ratios >25), indicating preferential channeling toward phenylalanine over tyrosine, potentially supporting auxin precursor biosynthesis or lignin/flavonoid pathways for cell wall remodeling during dedifferentiation. By Week 8, this

ratio normalized across all genotypes (ratios ~1.3-1.9), suggesting resolution of this metabolic bottleneck coincident with regeneration initiation.

The aspartate family metabolic shunt showed dramatic genotype- and week-specific redirections, with G12-Week 4 displaying exceptional elevation (ratio >450), indicating massive upregulation of aspartate family metabolism likely to meet methionine/SAM demands for epigenetic reprogramming during pluripotency acquisition. These temporal flux dynamics collectively define a three-phase metabolic model: Phase 1 (Week 4) characterized by metabolic priming and stress preparation, Phase 2 (Week 6) representing metabolic quiescence and reset, and Phase 3 (Week 8) showing coordinated pathway reactivation supporting regeneration competence, with each genotype employing distinct metabolic strategies to achieve this developmental transition.

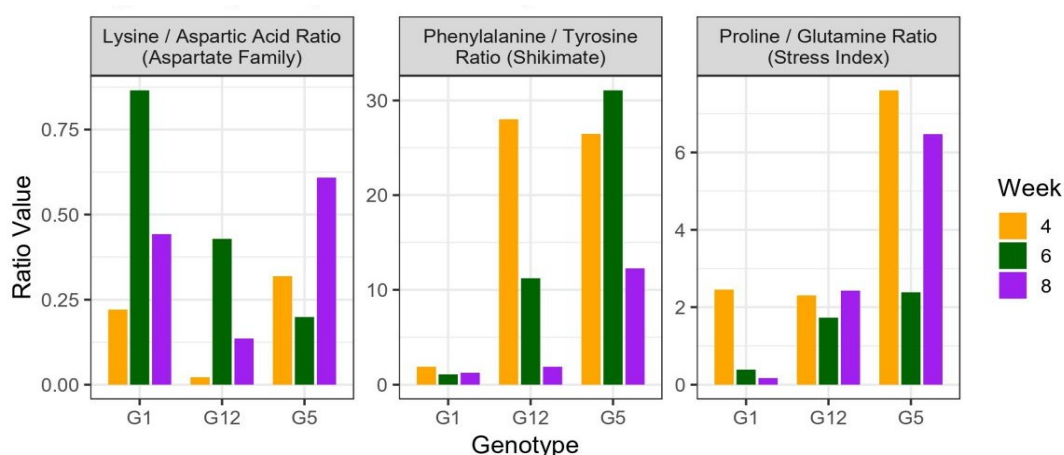


Figure 5. Time-series analysis of biosynthetic pathway flux ratios across three cocoa genotypes during callus development.

(A) Carbon-nitrogen balance ratio, (B) Stress/growth metabolite ratio, (C) Shikimate pathway flux ratio, and (D) Aspartate family metabolic shunt ratio. Data points represent means $\pm$ standard error ($n = 6$).

Discussion

Mobilisation of nutrients such as amino acids, proteins, lipids, polyphenols and carbohydrates in the culture medium is used by different biosynthesis pathways for embryo growth and development (Beguino *et al.*, 2024). In this study, a comparative approach was applied to study physiological differences among *T. cacao* explants-derived calluses with the hope to understand the complexity of the biosynthetic pathways that leads to embryogenesis for the genotypes under study. The concentration of free amino acids in cacao tissue depends on the ontogenic stage of the somatic embryos and the culture conditions (Beguino *et al.*, 2024). In the present study, amino acids profiles revealed differences among the genotypes examined and also differences among callus generated at different weeks. The results showed significant interactions between the genotype and week of the callus on embryogenic callus formation

(Mapayi *et al.*, 2024). This suggested the effect of genetic factors and physiological developments of the callus on embryogenic capacity of floral explants in *T. cacao* (Mapayi *et al.*, 2024; Moses and Asare, 2024).

Total phenols content was found to be different among the genotypes and the weeks of the callus. G5 was found to contain highest phenol content for callus generated at 8 weeks (Quiñones-Galvez and Martínez, 2020). In a similar study by Alemanno *et al.* (2003), flower buds of cacao contained different types of phenolic compounds, and each type was expressed qualitatively and quantitatively according to the developmental stage, indicating the key role played by these metabolites in the regulation of cell differentiation events. One of the factors generally considered as responsible for in vitro recalcitrance of cacao is the high polyphenol compounds content and their oxidation (Quiñones-Galvez *et al.*, 2016). In the current experiment, a high accumulation of these metabolites was particularly observed during callogenesis and embryo differentiation steps (at 8 weeks) (Quiñones-Galvez *et al.*, 2016). Hence, selection at this period for the genotypes under examination may result in poor embryo production due to coordinated pathway reactivation supporting regeneration competence being hindered by high phenolic oxidation.

Light, temperature, and plant nutrient availability influence polyphenol accumulation across plant tissues (Wang and Zheng, 2001; Piccaglia *et al.*, 2002). High accumulation of polyphenol compounds in floral explants during induction steps has been demonstrated to be unfavourable to somatic embryogenesis process (Quiñones-Galvez and Martínez, 2020). This observation was also made in date palm (*Phoenix dactylifera* L.) by Zouine and El Hadrami (2004).

Total sugar analysis revealed genotypic differences and variation across callus physiological development stages. G12 showed highest sugar content and optimal callus development at week 6. The significant genotype-week interaction highlights genetic factors and callus maturation's role in soluble sugar mobilization during embryogenesis (Mapayi *et al.*, 2024). Successful callogenesis correlates with elevated soluble sugar mobilization, supporting reducing carbohydrates' established role as key cell wall components essential for callus formation, cell differentiation, and osmotic regulation (Mapayi *et al.*, 2024).

Conclusion

The temporal profiling of metabolites during somatic embryogenesis in this study has revealed genotype dependent regulation of amino acid, phenolic, and soluble sugar dynamics during callus development. The results revealed coordinated pathway reactivation supporting regeneration competence, with each genotype employing distinct metabolic strategies to achieve this developmental transition. This suggested the effect of genetic factors and physiological developments of the callus on embryogenic capacity of floral explants in *T. cacao*.

Recommendations

The three-phase metabolic model, priming at Week 4, quiescence at Week 6, and pathway reactivation at Week 8, provides a clear temporal framework to guide subculture timing and medium adjustments in cocoa somatic embryogenesis (SE). Nitrogen and carbon supply should be modulated according to these developmental windows rather than applied uniformly.

Proline and cysteine peaks in G12 and G5 at Week 4 suggest their critical role in osmotic and oxidative stress management. Early supplementation with proline precursors and sulfur-containing compounds should be tested to boost embryogenic competence, particularly in recalcitrant genotypes.

Significant genotype x week interactions for glutamine, cysteine, and alanine indicate that medium formulations must be genotype-specific. Tailored adjustments, especially in nitrogen form and concentration, should be validated for G1, G5, and G12 to enhance SE efficiency.

The distinctive C/N ratio dynamics in G5 mark it as a priority for molecular studies aimed at identifying regulators of the callus to embryo transition. Coordinated management of phenolics and soluble sugars, particularly around Week 6, should also be considered, using interventions such as activated charcoal or polyvinylpyrrolidone to mitigate metabolic quiescence.

Validation across a broader panel of Nigerian cocoa accessions and under field relevant stress conditions is recommended to confirm the generality of the three phase model and establish metabolite guided criteria for selecting SE responsive genotypes.

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