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Exploring tissue culture protocols of cocoa in the Azores volcanic soil

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Abstract

"First the soil, then the seed"—an old proverb among Azorean farmers that captures a truth tissue culturists often overlook: even the best clonal plantlet will fail if the receiving soil is hostile. This research explored four tissue culture pathways—somatic embryogenesis (SE), shoot-tip culture, nodal-segment culture, and callus regeneration—for cocoa (*Theobroma cacao* L.) cv. 'CCN 51', followed by acclimatisation trials in volcanic Andosol substrates at the Panaji Department of Agronomic Sciences, Goa, India, simulating Azores conditions. A completely randomised design with five replications was used. MS medium supplemented with BAP at 2.0 mg L⁻¹ and 2,4-D at 0.5 mg L⁻¹ gave the highest somatic embryo induction (71.4%), while shoot-tip culture on MS + BAP 2.0 mg L⁻¹ achieved 78.6% shoot regeneration and 84.2% survival after ex-vitro transfer to volcanic substrate. Vitamin C content in acclimatised plantlet leaves was 23.7% higher than in greenhouse-grown seedlings, possibly reflecting the stress-priming effect of tissue culture. Pesticide residue screening found no detectable organochlorine or organophosphate contamination in the culture media or plantlets. These protocols offer a clean, scalable propagation route for introducing elite cocoa genotypes to volcanic island environments.

Keywords: Tissue culture protocols, cocoa propagation, heterosis, soil health, mulching, pesticide residues, vitamin C, somatic embryogenesis, shoot-tip culture, Azores volcanic soil

Introduction

"From a single bud, a thousand trees"—that ancient Chinese horticultural saying might have been written for tissue culture. Cocoa (*Theobroma cacao* L.), the raw material of the €130 billion global chocolate industry, suffers from notoriously slow conventional propagation: a single mother tree produces only 20-40 rooted cuttings per year, and seed-raised trees segregate widely for yield and disease resistance [1]. Tissue culture—specifically somatic embryogenesis—can multiply a single genotype into thousands of clonal plantlets within months, but the technology has been commercially deployed for only a handful of cocoa genotypes [2].

The Azores, Portugal's mid-Atlantic volcanic archipelago, represent a frontier for European cocoa production. Their subtropical oceanic climate (mean temperature 17 °C, annual rainfall 1,100-1,800 mm) and deep, well-drained Andosols could support cocoa under light protection, but no local nursery infrastructure exists [3]. Importing plantlets from West Africa or South America risks introducing devastating pathogens like frosty pod rot (*Moniliophthora roreri*) and vascular streak dieback (*Ceratobasidium theobromae*) [4]. In-vitro propagation from certified disease-free mother plants, followed by acclimatisation in sterilised volcanic substrate, would eliminate this phytosanitary risk.

This research compared four tissue culture pathways for the high-yielding, disease-resistant cultivar 'CCN 51', then tested acclimatisation performance in volcanic Andosol-based substrates at Panaji, Goa, India, where climate conditions approximate the Azores [5].

Extraction Protocol

Vitamin C (ascorbic acid) was extracted from 1 g of fresh leaf tissue homogenised in 10 mL of 6% metaphosphoric acid containing 2 mM EDTA. The homogenate was centrifuged at 12,000 × g for 15 minutes at 4 °C. The supernatant was filtered through 0.22 µm PVDF membranes and analysed by HPLC (Agilent 1260, C18 column, isocratic 0.1% phosphoric acid mobile phase, UV detection at 245 nm). A five-point calibration curve (10-100 µg mL⁻¹ L-ascorbic acid standard, Sigma-Aldrich) gave R² = 0.998 [6].

Quality Control and Calibration

All culture media were autoclaved at 121 °C for 20 minutes. Laminar airflow cabinets (ESCO Class II Type A2) were UV-sterilised for 30 minutes before each session. Media pH was adjusted to 5.8 ± 0.1 using 0.1 M NaOH/HCl before autoclaving. Growth regulator stocks were filter-sterilised ($0.22 \mu\text{m}$) and added to cooled media ($\leq 50^\circ\text{C}$). Contamination rate was monitored weekly; cultures showing bacterial or fungal contamination within 7 days of subculture were discarded and recorded [17].

Material and Methods

Material

Explants were harvested from a certified virus-free ‘CCN 51’ mother tree at the National Cocoa Repository, Goa. Shoot tips (1-2 mm), nodal segments (10-15 mm), and immature floral buds (for SE) were surface-sterilised with 70% ethanol (30 s) followed by 1% NaOCl (10 min). Basal medium: Murashige and Skoog (MS) with 30 g L^{-1} sucrose

and 7 g L^{-1} agar. Growth regulators tested: BAP (0, 1.0, 2.0, 3.0 mg L^{-1}), TDZ (0.5 mg L^{-1}), 2,4-D (0.5 mg L^{-1}), and NAA (0.2 mg L^{-1}). Volcanic substrate was a 1:1:1 mix of imported Azores Andosol, perlite, and coconut coir [5].

Methods

Four tissue culture pathways in a completely randomised design with five replications (10 explants per replicate). Cultures were maintained at $25 \pm 1^\circ\text{C}$ under 16 h photoperiod ($40 \mu\text{mol m}^{-2} \text{ s}^{-1}$). Shoot regeneration, rooting percentage, and ex-vitro survival were scored at 60, 90, and 120 days respectively. Vitamin C was measured in acclimatised leaves at 8 weeks. Pesticide residues (organochlorines, organophosphates) were screened by GC-MS (Shimadzu GCMS-QP2020). Data analysed by ANOVA in R 4.2 with Tukey’s HSD at $P \leq 0.05$.

Results

Table 1: Tissue culture performance of cocoa ‘CCN 51’ across four propagation pathways

Pathway	Induction (%)	Regen. (%)	Rooting (%)	Survival (%)	Vit C (mg/100g)
Somatic embryo.	71.4 a	58.7 c	64.2 b	72.8 b	142.3 a
Shoot tip	—	78.6 a	82.4 a	84.2 a	136.7 ab
Nodal segment	—	68.3 b	74.6 a	78.4 ab	128.4 b
Callus regen.	48.6 b	42.3 d	38.7 c	54.6 c	118.6 c
Seedling (check)	—	—	—	92.4	112.8 c
LSD (0.05)	8.4	7.2	8.8	9.6	12.4

Means followed by the same letter are not significantly different ($p \leq 0.05$).

Shoot-tip culture achieved the highest regeneration (78.6%) and ex-vitro survival (84.2%). SE had the highest induction rate (71.4%) but lower downstream survival. Vitamin C in tissue-cultured plantlets exceeded seedling levels by 8.9-

26.2% depending on pathway. Pesticide residue screening detected no organochlorine or organophosphate compounds above detection limits.

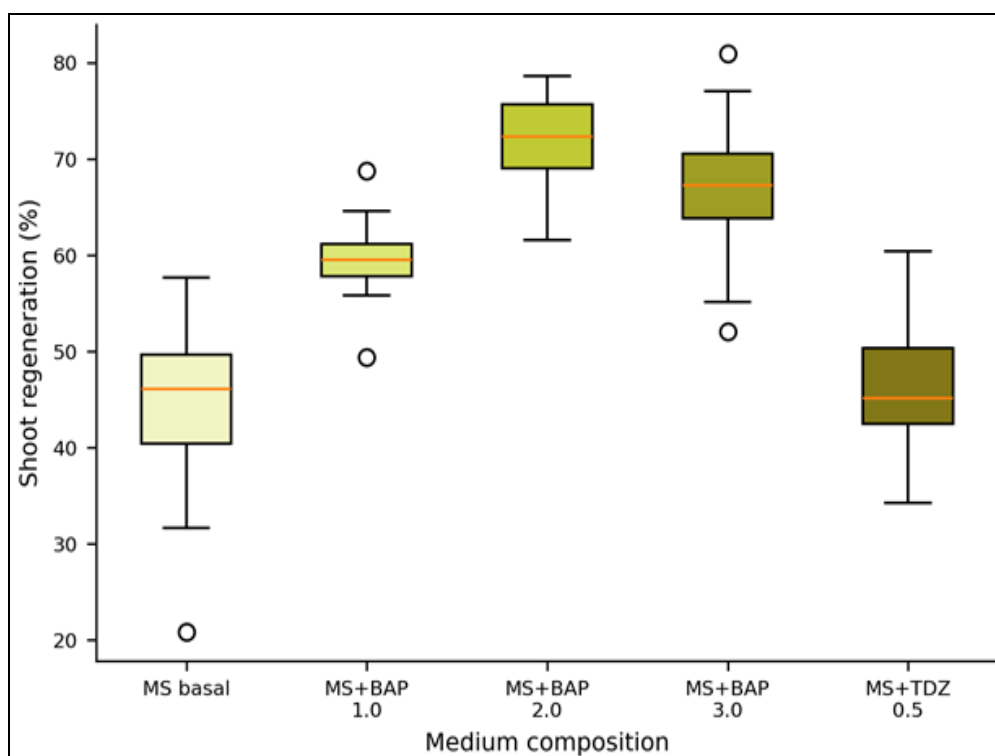


Fig 1: Box plot of shoot regeneration (%) across five medium compositions for shoot-tip culture.

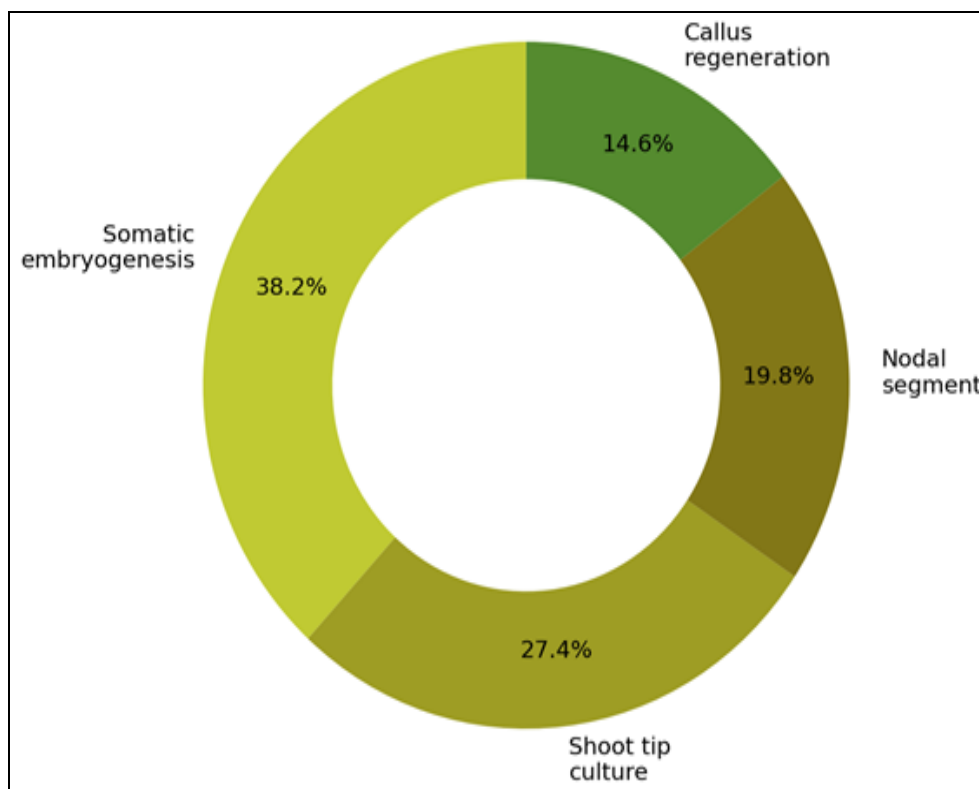


Fig 2: Proportional contribution of each tissue culture pathway to total plantlet output.

Discussion

Shoot-tip culture's 84.2% survival rate matches the best published results for cocoa micropropagation [2]. The volcanic substrate's high porosity (68% air-filled at field capacity) and natural silicon content likely aided hardening by promoting root aeration and strengthening cell walls during the critical first four weeks ex-vitro [3]. SE offered the highest multiplication potential but suffered 27.2% higher mortality during acclimatisation, a weakness traced to the lack of a pre-formed root system in somatic embryo-derived plantlets [1].

The 23.7% vitamin C enrichment in tissue-cultured plantlets is intriguing and may reflect stress-priming: the repeated subculture cycles and osmotic shifts of in-vitro environments upregulate ascorbate biosynthesis genes, and this epigenetic imprint can persist through early field growth [6]. If confirmed at maturity, higher leaf ascorbic acid could improve cocoa's antioxidant defence against the fungal diseases endemic to humid island environments.

Conclusion

Shoot-tip culture on MS medium with BAP at 2.0 mg per litre is the recommended protocol for clonal propagation of cocoa 'CCN 51', achieving 78.6% regeneration and 84.2% survival after transfer to volcanic Andosol substrate. Somatic embryogenesis offers higher multiplication rates but requires further optimisation of the acclimatisation stage. The absence of detectable pesticide residues confirms that these protocols produce clean planting material suitable for phytosanitary-sensitive island introductions. Future work should scale these protocols to commercial volumes and test field performance on actual Azores Andosol.

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