



## Research Note

# Genetic assessment of *in vitro* propagated *Coffea arabica* plants using RAPD makers

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### Abstract

To cultivate plants that accurately reflect the original variety, it is necessary to monitor the variation that occurs, to select plants with true-to-type characteristics and reduce variability. In this study, *in vitro* propagation of (*Coffea arabica* var. Catimor) plants was adopted to generate large number of disease-free coffee plants within a short period. Genetic variability among the tissue culture derived plants in compared to the mother plant was assessed at molecular level with RAPD markers. For the tissue culture experiment, the optimal sterilization was treatment with 15 % Clorox for 15 minutes, which resulted in an 80 % survival rate and a 26.7 % germination rate. The MS medium supplemented with 1 mg/l of the growth regulator, 6-benzylaminopurine, was observed to produce the best growth. Screening of the tissue cultured plantlets and mother plants with seven selected RAPD primers revealed no significant genetic variation.

**Keywords:** Tissue culture, Coffee, RAPD markers

Plant tissue culture is a widely used method in plant propagation that facilitates large-scale, quick plant multiplication regardless of the season or environmental factors. Additionally, this method aids in the propagation of rare or endangered species or plants that struggle with natural reproduction. It is also used to produce and increase the quantity of significant chemicals or secondary metabolites from plants. The tissue culture technique has been applied to propagate various types of coffee plants, such as *Coffea arabica* (Ebrahim *et al.*, 2007; Abd El Gawad *et al.*, 2012) and *Coffea canephora* (Robusta) (Da Silva and Dos Santos, 2022 and Santana *et al.*, 2004). This research aims to study tissue culture techniques for the propagation of *Coffea arabica* var. Catimor, which is widely cultivated and serves as the main coffee crop in the Pang Khon region, Chiang Rai Province, Thailand. There are several limitations in coffee cultivation. One identified aspect is the quality of coffee seedlings, leading to final yields that do not meet the required quality. This research addresses this problem through a tissue culture

approach to generate disease-free coffee plants in large quantities within a short period.

Various factors can lead to changes in the cellular structure of progeny plants. As these plants develop, they may exhibit characteristics that differ from the original. Such traits can be inherited by subsequent generations. This variation poses a significant problem in plant propagation when the goal is to produce plants with consistent traits. Therefore, it is necessary to examine the variation in order to select plants that match the desired traits and to reduce unwanted variation. Random DNA extraction from living organisms is a crucial step for the study of molecular genetics. Once DNA is extracted in sufficient quantity and of suitable quality, it can be used to analyze variations in DNA patterns using various molecular marker techniques. Moreover, Random Amplified Polymorphic DNA (RAPD) is a method used to analyze genetic variation. These techniques are essential for plant variety identification or the analysis of genetic

variation, as morphological characteristics alone may not clearly distinguish differences.

When the goal is to produce plants that accurately reflect the original, it is necessary to monitor the variation that occurs in order to select plants with true-to-type characteristics and reduce variability. Currently, molecular markers are commonly used to detect differences at the DNA level. RAPD markers have been applied to assess variation in many plant species. Therefore, genetic identity of the tissue-cultured plants can also be verified using RAPD markers. The present study was undertaken with an objective of evaluating tissue culture-derived *C. arabica* var. Catimor plants using RAPD molecular markers by comparing their DNA profiles with those of the mother plants.

**In vitro propagation of Coffee:** Shoot tips and seeds from *C. arabica* variety Catimor were used as explants for the study. Fresh seeds collected from healthy, mature parent plants were classified into two groups: unripe green seeds and ripe red seeds. Shoot tip explants of approximately 0.5–1.0 cm in length were excised from young, actively growing seedlings. Explants were washed thoroughly under running water for 10–15 minutes to remove surface contaminants. The samples were then surface sterilized by immersion in 10% and 15% Clorox for 15 minutes, followed by four rinses with sterile distilled water under aseptic conditions to remove residual disinfectant. Sterilized explants were inoculated onto Murashige and Skoog (MS) basal medium supplemented with different concentrations of the 6-benzylaminopurine (BA). The treatments consisted of MS alone (control), MS + 1 mg/L BA, MS + 2 mg/L BA, and MS + 3 mg/L BA. The culture medium was supplemented with 3 % (w/v) sucrose as a carbon source and solidified with 0.8% (w/v) agar. The pH of the media was adjusted to 5.8 prior to autoclaving and it was sterilized by autoclaving at a temperature of 121°C and pressure of 15 psi for 15 minutes. After inoculation, the cultures were incubated at 25 ± 2°C under a 16-hour photoperiod with a light intensity of approximately 40–60  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The explants were monitored regularly for contamination and growth responses. Subculturing was

carried out at four-week intervals onto fresh media of the same composition to promote plant development and proliferation.

**DNA barcoding:** DNA from mother coffee plants and tissue culture-derived plants were extracted using the GF Plant DNA Extraction Kit. The target region, namely, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*), was amplified by Polymerase Chain Reaction (PCR), with details of the primers and experimental conditions as furnished in **Table 1**. The DNA was purified for further nucleotide sequence analysis. The obtained sequences were subsequently analyzed and processed using the FinchTV program.

**Genetic variation analysis using RAPD technique:** The extracted DNA from plant samples was amplified by PCR with 19 RAPD primers specific to *rbcL* for primer screening (OPAV11, OPH15, OPAG02, OPAY03, OPAA14, OPAW18, OPAW03, OPZ12, OPAW17, OPN17, OPAQ13, OPI19, OPAQ06, OPX11, OPAP09, OPAT13, OPAI12, OPX03, and OPC06). The total reaction volume was 50  $\mu\text{l}$ , containing 1  $\mu\text{l}$  KOD-Plus-Neo DNA polymerase, 5  $\mu\text{l}$  10X buffer, 5  $\mu\text{l}$  2 mM dNTPs, 3  $\mu\text{l}$  25 mM  $\text{MgSO}_4$ , 3  $\mu\text{l}$  primers, 1  $\mu\text{l}$  DNA, and 32  $\mu\text{l}$   $\text{H}_2\text{O}$ . For the PCR process, the initial temperature was set at 94°C for 2 minutes, followed by denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 30 seconds per kilobase. After 35 cycles, the final extension step was conducted at 72°C for 10 minutes. The PCR product was size fractionated by electrophoresis on a 1.5% agarose gel in TBE buffer (Tris Base, Boric acid,  $\text{Na}_2\text{EDTA}$  0.5 M, pH 8.0) at 50 V for 60 minutes. The DNA bands obtained from the mother plants and the tissue culture-derived plants were compared to assess genetic variation.

**Coffee propagation using tissue culture technique:** The efficacy of the sterilization treatments was first evaluated by comparing the contamination and survival rates of the treatment. The results showed that shoot explants exposed to all tested concentrations of Clorox were ineffective, as 100 % contamination was observed and

**Table 1. PCR protocol for *rbcL* amplification**

Component	Volume ( $\mu\text{L}$ )	Final concentration
10× KOD-Plus-Neo buffer	5	1x
2mM dNTPs	5	0.2 mM each
25mM $\text{MgSO}_4$	3	1.5 mM
10 pmol/ $\mu\text{L}$ Primer #1	1.5	0.15 $\mu\text{M}$
10 pmol/ $\mu\text{L}$ Primer #2	1.5	0.15 $\mu\text{M}$
Template DNA	1	Genomic DNA $\leq$ 200 ng/50 $\mu\text{L}$
PCR grade water	32	-
KOD-Plus-Neo DNA polymerase (1.0 U/ $\mu\text{L}$ )	1	1.0 U / 50 $\mu\text{L}$
Total reaction volume	50	-

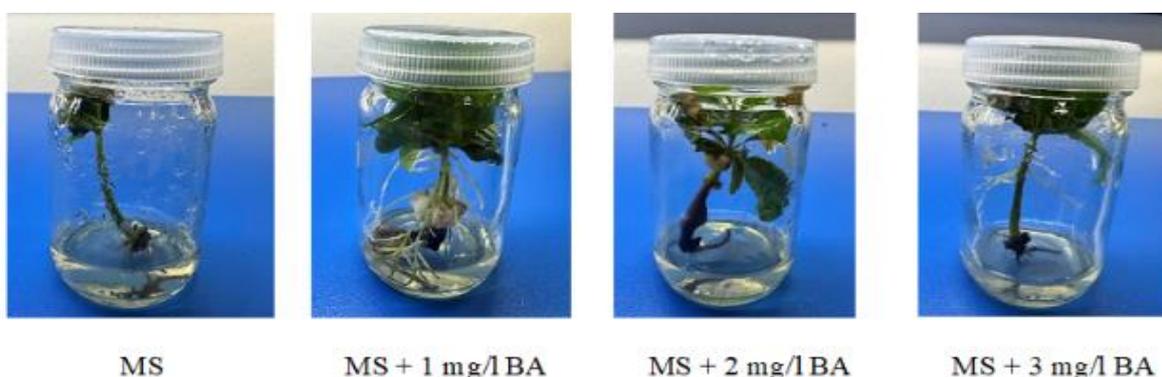
**Table 2. Survival rate and germination rate of Arabica coffee seeds, Var. Catimor**

Explant types	Sterilization treatment	Survival rate (%)	Germination (%)
Shoot tips	10 % Clorox, 15 min	0	0
	15 % Clorox, 15 min	0	0
Seeds	Ripe coffee seeds		
	10 % Clorox, 15 min	86.7	0
	15 % Clorox, 15 min	80.0	26.7
	Unripe coffee seeds		
	10 % Clorox, 15 min	70	10
	15 % Clorox, 15 min	90	10

no shoot explants survived. This failure was attributed to persistent fungal contamination associated with shoots collected from naturally grown mother plants, which could not be eliminated through the sterilization treatments. In contrast, seed explants responded more favorably to sterilization and culture conditions. All media formulations supported seed germination and seedling development, with the survival and germination rates presented in **Table 2**. These findings are consistent with previous reports indicating that plant seeds are suitable explants for tissue culture due to their ease of sterilization and lower risk of contamination (Choengpanya *et al.*, 2021; Sritapanya and Chotikadecha, 2021 and Chumphukham, *et al.*, 2016). The result showed that induction with MS medium supplemented with 1 mg/l BA yielded the highest growth of both roots and leaves, as shown in **Plate 1**. The optimal sterilization formula was 15 % Clorox for 15 minutes, which produced an 80 % survival rate and a 26.7 % germination rate. The seedlings were transferred to a medium supplemented with plant growth regulators. The results showed that MS medium + 1 mg/l BA yielded the highest growth, making it suitable for propagation of Catimor coffee. Furthermore, examination of tissue-cultured plants in comparison with the natural mother plants revealed no detectable difference.

**DNA barcode results:** In addition to morphological identification, DNA barcoding was conducted to confirm the species identity of the coffee samples. DNA barcoding using the *rbcl* gene was employed to confirm the

taxonomic identity of the plant material prior to genetic variability analysis, thereby ensuring that all samples analyzed in this study belonged to the same species. While there are several intriguing DNA regions, such as the internal transcribed spacer (ITS), *rbcl*, *trnL-F* intergenic spacer, *psbA-trnH* intergenic spacer, *matK*, *ndhF*, and *atpB*, the ribulose biphosphate carboxylase (*rbcl*) gene was selected as a marker in this research. It encodes a subunit of the enzyme ribulose biphosphate carboxylase oxygenase (RUBISCO), which is involved in the fixation of CO<sub>2</sub> during photosynthesis in plants. It is widely used as a marker for studying evolutionary relationships among many plant species (Schneider and Schuettpelz, 2006). PCR amplification of the chloroplast ribulose-1,5-biphosphate carboxylase/oxygenase large subunit (*rbcl*) gene was successfully performed for all samples. Electrophoretic analysis of the PCR products revealed a single DNA band of approximately 550 bp, indicating successful amplification of the target fragment. The amplified PCR products were purified and subjected to DNA sequencing using the Sanger sequencing method. The resulting nucleotide sequences of the *rbcl* gene were compared with reference sequences available in the NCBI GenBank database using BLAST analysis (Altschul *et al.*, 1990) to verify the species identity of the samples. The sequence analysis confirmed that both samples corresponded to *Coffea arabica*. Moreover, multiple sequence alignment using the Clustal Omega tool showed no nucleotide differences between the two *rbcl* sequences obtained in this study (**Fig. 1**).

**Plate 1. Coffee seedlings grown under sterile conditions on different media formulations**

**Genetic variation analysis:** The tissue culture plantlets were verified in comparison to the original mother plants using RAPD markers. Genomic DNA was extracted from fresh leaves of both the mother plants and the tissue culture–derived progeny plants, and DNA quality was assessed by agarose gel electrophoresis. PCR amplification was initially performed using a set of 19 RAPD primers to screen for primers capable of producing clear and reproducible amplification profiles. The amplified PCR products were separated by electrophoresis on an agarose gel, and the DNA banding patterns were visualized under ultraviolet light using a gel documentation system. Based on the screening results, seven primers namely, OPAG03, OPAH17, OPAV11, OPC06, OPAH15, OPAQ06, and OPX11, were identified for further analysis. Genetic variation between the tissue culture–derived plants and the mother coffee plants were assessed based on the above primers. PCR amplification with primers OPAG03, OPAH17, OPAV11, OPC06, OPAH15, and OPAQ06 generated clear and scorable DNA bands following agarose gel electrophoresis. These primers produced a total of 5, 1, 11, 8, 6, and 9 bands, respectively. The banding patterns obtained from the tissue culture–derived plants were identical to those of the mother plants, and were monomorphic (**Fig.2**). In contrast, amplification with primer OPX11 produced bands that lacked clarity to allow reliable scoring of polymorphism.

RAPD markers are commonly used to confirm that tissue culture plants are genetically identical to the parent plant (Suresh *et al.*, 2013). Currently, molecular markers are widely used to detect genetic variation at the DNA level in plants propagated through tissue culture. RAPD markers have been applied to assess variation in many plant species, such as coffee (Babu *et al.*, 2007 and Vidhya *et al.*, 2016), ginger (Rout *et al.*, 1998), rubber tree (Sirisom and Techato, 2013), lemongrass (Dey *et al.*, 2015), Nepenthes (Devi *et al.*, 2013) and oil palm (Athipattjaporn and Techato, 2012). In the present study, the identity of the tissue-cultured *C. arabica* var. Catimor, with the mother plants, is validated using RAPD markers, ensuring that the clones maintain desirable traits and that no significant genetic variation has occurred during the *in vitro* propagation process. Even when the plants are still immature, RAPD markers offer a convenient, simple, rapid, and accurate method to analyze DNA because they don't require prior knowledge of the DNA base sequence of the plants being studied (Piyachoknakul, 2009).

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BCoff_RBC	TCAGCTGGTGTAAAGAGTACAAATTGACTTATTATACTCCTGAATACGAAACCAAAGATA	60
SCoff_RBC	TCAGCTGGTGTAAAGAGTACAAATTGACTTATTATACTCCTGAATACGAAACCAAAGATA	60
BCoff_RBC	CTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCCGGGAGTTCCACCTGAAGAAGCGG	120
SCoff_RBC	CTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCCGGGAGTTCCACCTGAAGAAGCGG	120
BCoff_RBC	GGGCCGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAGCTGTGTGGACCGATGGGC	180
SCoff_RBC	GGGCCGCGGTAGCTGCCGAATCTTCTACTGGTACATGGACAGCTGTGTGGACCGATGGGC	180
BCoff_RBC	TTACCAGCCTTGATCGTTACAAAGGGCGATGCTATCACATCGAGCCAGTTCCTGGGGAAG	240
SCoff_RBC	TTACCAGCCTTGATCGTTACAAAGGGCGATGCTATCACATCGAGCCAGTTCCTGGGGAAG	240
BCoff_RBC	AAAATCAATATATTGCTTATGTAGCTTACCCCTTAGACCTTTTGAAGAAGGTTCTGTTA	300
SCoff_RBC	AAAATCAATATATTGCTTATGTAGCTTACCCCTTAGACCTTTTGAAGAAGGTTCTGTTA	300
BCoff_RBC	CTAACATGTTTACTTCCATTGTAGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTAC	360
SCoff_RBC	CTAACATGTTTACTTCCATTGTAGGTAATGTATTTGGGTTCAAAGCCCTGCGCGCTCTAC	360
BCoff_RBC	GTCTGGAAGATTGCGAGTCCCACCTGCTTATATTAACCTTCCAAGGGCCGCTCATG	420
SCoff_RBC	GTCTGGAAGATTGCGAGTCCCACCTGCTTATATTAACCTTCCAAGGGCCGCTCATG	420
BCoff_RBC	GCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCTGTTGGGATGTA	480
SCoff_RBC	GCATCCAAGTTGAGAGAGATAAATTGAACAAGTATGGTCGTCCTGTTGGGATGTA	480
BCoff_RBC	TTAAACCTAAATTAGGTTTATCTGCTAAAACTATGGTAGAGCTGTTTATGAATGTCT	538
SCoff_RBC	TTAAACCTAAATTAGGTTTATCTGCTAAAACTATGGTAGAGCTGTTTATGAATGTCT	538

**Fig. 1. Comparison of the rbcL gene sequence from BCoff\_RBC and SCoff\_RBC**

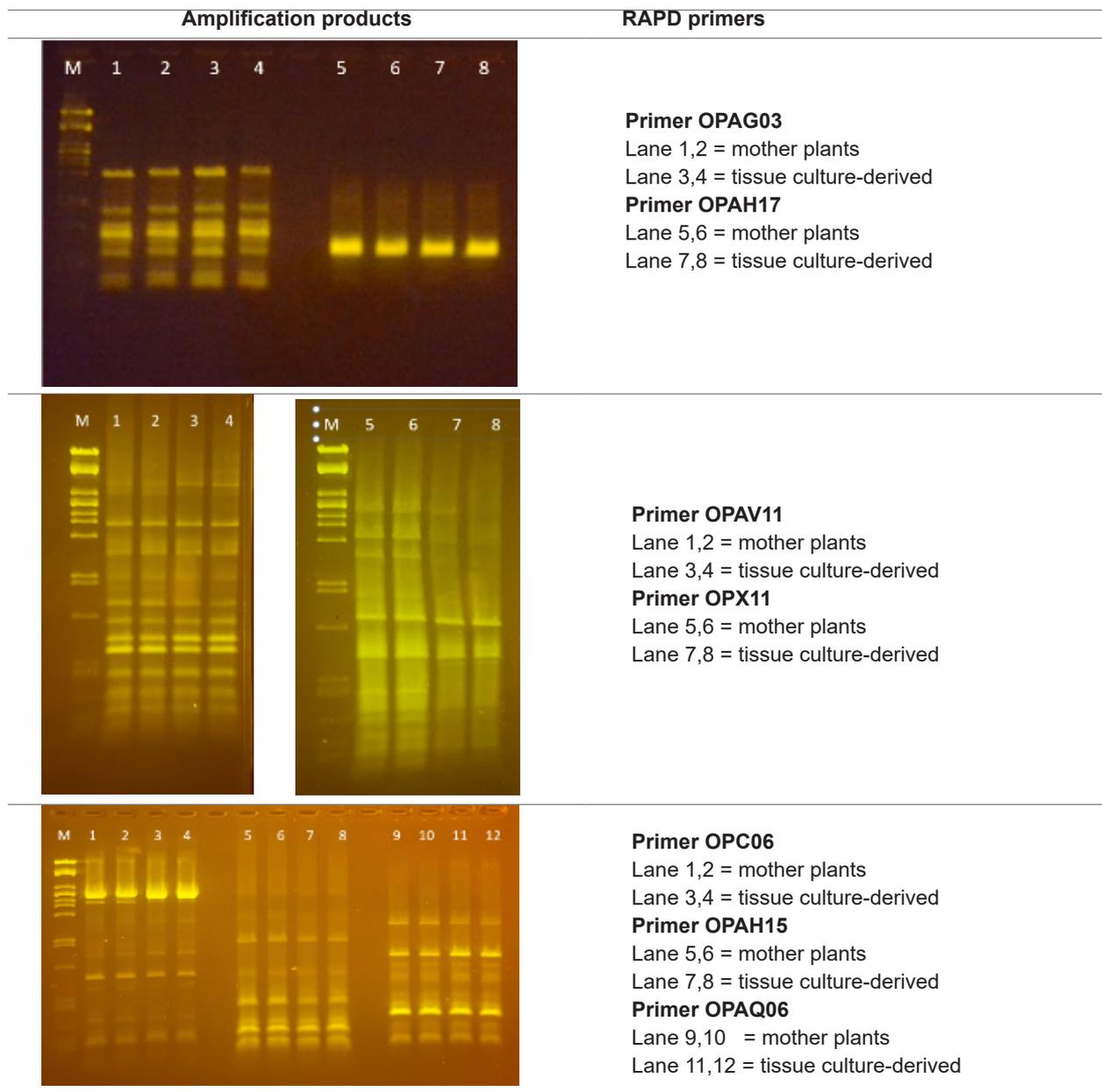


Fig. 2. DNA banding patterns produced by RAPD markers

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