Journal of Current Research in Engineering and Science
Bi-Annual Online Journal (ISSN: 2581 - 611X)

VOLUME:9, ISSUE:1, JANUARY 2026, PAPER NUMBER:4

RAPD Based Genetic Differenciation and Disease Resistance Evaluation of Hevea brasiliensis clones

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Abstract: *Rubber tree (Hevea brasiliensis), the principal source of natural rubber, plays a vital role in various industrial applications. Despite the economic value of high-yielding clonal cultivars such as RRII105 and PB260, the uniform genetic makeup of these clones increases vulnerability to pathogens, especially Corynespora cassiicola, the causal agent of Corynespora Leaf Fall Disease (CLFD). The development of disease-resistant clones is thus essential for sustainable rubber production. This study investigates genetic variation between RRII105 and PB260 using Random Amplified Polymorphic DNA (RAPD) analysis and evaluates their relative resistance to CLFD through a virulence assay. DNA was isolated from each clone using a modified CTAB method (Doyle & Doyle, 1987) [1], optimized with β-mercaptoethanol for improved pigment removal. Seven RAPD primers were used to amplify genomic DNA, revealing polymorphic bands indicative of genetic diversity. The percentage of polymorphism ranged from 6.25% to 10.45%, with RRII105 showing slightly more diversity. Notably, primer-specific bands unique to PB260 may correlate with phenotypic traits. Virulence testing with five fungal isolates showed that RRII105 developed smaller lesions than PB260, suggesting better resistance to CLFD. These differences support the hypothesis that genetic polymorphism contributes to differential disease responses.*
Keywords: *Hevea brasiliensis, RRII105, PB260, CLFD, RAPD, virulence assay, primers, genetic diversity.*

1. INTRODUCTION:

Natural rubber, derived predominantly from *Hevea brasiliensis*, underpins a global industry reliant on tire manufacturing, medical devices, and countless industrial products (Priyadarshan, 2011) [2]. With commercial cultivation centered on genetically uniform clones like RRII105 and PB260, the risk of epidemic disease outbreaks is substantial. In recent years, Corynespora Leaf Fall Disease (CLFD), caused by *Corynespora cassiicola*, has emerged as a serious threat, leading to significant yield losses in Asia (Jayasinghe & Fernando, 2009; Yu et al., 2022) [3]. To address this challenge, evaluating intra-clonal genetic variation and resistance traits is critical. Random Amplified Polymorphic DNA (RAPD) markers have become a favored tool due to their simplicity and effectiveness in detecting genome-wide variation (Williams et al., 1990) [4]. RAPD enables the identification of clonal divergence, aiding in germplasm management, breeding selection, and trait mapping (Mantello et al., 2014). The current study applies RAPD analysis to assess the genetic variability of RRII105 and PB260 clones from different sources and correlates these differences with resistance to CLFD. DNA extraction followed the Doyle & Doyle (1987) protocol, with modifications to enhance purity for successful amplification. Seven primers were selected for RAPD analysis, and the resultant banding patterns were used to calculate polymorphism percentages. To complement the molecular approach, a virulence assay using five *C. cassiicola* isolates was conducted. Lesion development was measured on leaf discs of each clone over a 92-hour incubation period. The comparative lesion diameters provided phenotypic validation of the molecular results.

RRII105 displayed relatively smaller lesions, suggesting increased resistance compared to PB260. This integrative approach offers a framework for identifying resistant clones using molecular tools and bioassays.

RAPD

Random Amplified Polymorphic DNA (RAPD) is a PCR-based DNA fingerprinting technique that uses short, arbitrary primers (typically 10 nucleotides long) to amplify random segments of genomic DNA (Williams et al., 1990). Unlike traditional PCR, RAPD does not require prior knowledge of the DNA sequence, making it a cost-effective and rapid method for genetic analysis. The primers bind to complementary sequences at multiple sites on the DNA template, and the amplification products are separated by gel electrophoresis to produce a unique banding pattern for each individual or clone (Saha and Roy, 1992). Williams et al. (1990) introduced the RAPD technique, demonstrating its utility in generating polymorphic DNA markers for genetic mapping and diversity studies. Saha and Roy (1992) applied RAPD markers to study genetic diversity in *H. brasiliensis*, successfully distinguishing closely related clones. Venkatachalam et al. (2002) used RAPD markers to assess genetic variability among rubber tree clones, identifying unique genetic fingerprints for several clones. RAPD markers are dominant, meaning they detect the presence or absence of a band rather than distinguishing between homozygous and heterozygous states (Lowe et al., 2000). This technique is widely used for assessing genetic diversity, identifying genetic markers, and distinguishing closely related species or clones (Venkatachalam et al., 2002). However, RAPD has limitations, including low reproducibility due to sensitivity to PCR conditions and the dominance of markers, which can complicate data interpretation. Despite these limitations, RAPD remains a valuable tool for preliminary genetic screening and diversity studies in plants, including *Hevea brasiliensis* (Souza et al., 2008).

2. MATERIALS AND METHODS

Materials

Freshly plucked leaves of 2 different clones of Hevea brasiliensis each sampled from three places, Genome lab, Rubber Research Institute of India (RRII105-1 and PB260-1), Budwood nursery (RRII105-2 and PB260-2) and Botany field (RRII105-3 and PB260-3).

Reagents

- CTAB (Cetyltrimethylammonium bromide)
- Beta-mercaptoethanol (5%)
- Ethanol (70%)
- 24:1 Chloroform:isoamyl alcohol

The random primers (OPAE12, OPAI6, OPAI9, OPAD12, OPAB7, OPAB14, OPG17), Taq DNA polymerase, dNTPs and RNAase A enzyme were from Takara Bio India Pvt. Ltd.

Methods

DNA Extraction and Purification

The DNA extraction and purification method was conventionally adopted from Doyle JJ and

Doyle JL (1987) with some improvisations as referred. *H. brasiliensis* leaves were plucked afresh from various fields as mentioned earlier. 0.8 grams of leaves was weighed and ground rapidly in liquid nitrogen. To these finely powdered leaves sample, 5ml of preheated (60°C) 2% CTAB solution with betamercaptoethanol and was added and mixed well. This viscous mixture is transferred to a 50 ml polypropylene centrifuge tube. Then, this tube was incubated for 45 minutes at 60°C with intermittent

mixing by inverting the tube. After cooled down to room temperature, equal volume of 24:1 Chloroform:isoamyl alcohol was added, mixed several times by inverting the tube, and centrifuged at 10,000 rpm for 10 minutes. The DNA samples were quantified using spectrophotometer and made upto 10ng/µl.

RAPD PCR

7 random primers were used for effective result interpretations. The sequences of the primers are listed in Table 1.1. For each primer, PCR was repeated until the best gel image produced. The reaction mixture (10µl) of PCR had contained 2µl sample DNA, 5.9µl milliQ water, 1µl buffer 0.2µl dNTPs, 0.8µl primer solution and 0.1µl Taq polymerase. After adding all these solutions in precise quantities with the 6 extracted DNA samples each, PCR was run. PCR cycle temperature and durations were customized in an Eppendof Thermal Cycler as follows:

PCR SYSTEM:

Initial Denaturation : 4 minutes at 95°C
Cycle starts
Denaturation : 30 seconds at 94°C
Annealing: 1 minute at 36°C
Extension: 2 minutes at 72°C
Cycle ends
Final Extension: 10 minutes at 72°C

Virulence Test using Fungal Disc Method

5 isolates of *Corynespora cassiicola* fungus were cultured on Potato Dextrose Agar (PDA) plates for 5-7 days at 28°C. From the actively proliferating margin of the culture, fungal discs were punched out using sterile cork borer. Fresh, mature and washed rubber leaves of RRII105 and PB260 were placed in sterile and moist Petri dishes. Fungal discs were cut and placed on the adaxial side of the leaves with gentle pressure. The dishes were then incubated at room temperature for 72 hours and observed the lesions. The diameter of the lesions was marked and analyzed.

3. RESULTS AND ANALYSIS

Extraction and purification of leaf total DNA of Hevea brasiliensis

As a preparative experiment, the leaf total DNA of two clones of *Hevea brasiliensis* from three different sources were isolated using the method of Doyle JJ and Doyle JL (1987). Polyphenols and polysaccharides, however, frequently compromise the integrity of DNA extracted from plants (Porebski et al., 1997) [6]. This was addressed by adding 5% β-

mercaptoethanol, which effectively removed pigment impurities and produced high-quality DNA that could be amplified using PCR. DNA was extracted successfully upon the addition of 70% ethanol and air dried. DNA samples from RRII105 and PB260 from three different sources were obtained and quantified using a spectrophotometer. Analysing the absorbance at 260 nm allowed for the determination of the isolated DNA concentrations. By diluting the pellet with sufficient amount of milliQ water, the final concentrations of each sample were brought down to 10ng/µl for RAPD analysis.

RAPD analysis

After careful addition of the compositions of the reaction mixture along with the DNA samples, 7 rounds of RAPD analysis were performed for 7 primers in a PCR system with the customized temperature cycles as mentioned earlier. Each round was followed by gel electrophoresis on 1% Agarose gel with 80V power electric current for about 30 minutes. DNA bands were observed and photographed using *Gel doc - Vilber*. The photographed gel images are shown in Figures 1. The number of total RAPD loci, and polymorphic DNA loci were calculated. The percentage polymorphism obtained for each clone is mentioned in Table 1.

Sl. No.	**Amplified RAPD locii**							
	Primers	***Sequence***	***RRII1051***	***RRII105 2***	***RRII105 3***	***PB260 1***	***PB260 2***	***PB260 3***
1.	OPAE12	CCGAGCAATC	11	11	11	12	12	12
2.	OPAI6	TGCCGCACTT	10	10	10	9	10	10
3.	OPAI9	TCGCTGGTGT	11	9	9	9	9	9
4.	OPAD12	AAGAGGGCGT	8	8	8	8	8	8
5.	OPAB7	GTAAACCGCC	9	9	9	8	5	7
6.	OPAB14	AAGTGCGACC	8	8	8	8	8	8
7.	OPG17	ACGACCGACA	10	10	10	12	12	12

Table 1: RAPD Amplification Result

A total of 66–67 RAPD loci were amplified in RRII105 and 64–66 in PB260 across different sources. The percentage of polymorphic loci ranged from 7.57% to 10.45% in RRII105 and 6.25% to 9.09% in PB260. These figures suggest low to moderate genetic diversity within each clone, possibly arising from somaclonal variations or source-specific environmental factors. Notably, certain primers (e.g., OPAE12 and OPG17) amplified bands uniquely in PB260, indicating genotype-specific sequences that may underlie phenotypic traits like latex yield or stress response (Souza et al., 2009). These genetic differences, although subtle, are valuable for clone identification and management in breeding programs. The polymorphic loci represent genomic regions that could be further sequenced to identify genes of interest related to agronomic traits. The comparative analysis on the RAPD locii numbers suggests that there is a considerable difference in the genetic profile of both the clones. One band was polymorphic and exclusively seen in PB260 for OPAE12 and OPG17 primers. These more

RAPD loci can be a genetic sequence that may enhance or add to the existing traits of H. brasiliensis such as high yield, disease resistance, etc.

Virulence Test Analysis

The virulence test aimed to assess susceptibility differences between the two clones when exposed to *Corynespora cassiicola*, the causative agent of Corynespora Leaf Fall Disease (CLFD). The disease is one of the major threats to rubber production, especially in Asia, and resistance breeding is crucial for sustainable cultivation (Jayasinghe and Fernando, 2009). 5 different isolates of *Conrynespora cassiicola* were used for the analysis of virulence. A total of 16 discs of each isolate were placed on 3 leaves of each clone (RRII105 and PB260). It was noted that distinct circular lesions were developing during the long incubation of RRII105 and PB260 leaves with fungal discs. The lesions initially appeared as small,

circular spots further developed to become irregular, zonate concentric rings. They were dark brown and even black at the centers. As the lesions matured, the center part turned greyish or brittle. Lesion diameters (in cm) after 92 hours of incubation was measured using a scale. Five fungal isolates were tested on 16 leaf discs per clone. After 92 hours of incubation, lesion development patterns were recorded as mentioned in Table 2. RRII105 consistently showed slightly smaller lesion diameters compared to PB260, with an overall average difference of 2.875 cm (Table 3). Lesions began as small circular spots and evolved into large, irregular necrotic areas with brittle centers, consistent with typical CLFD symptoms (de Souza et al., 2017).

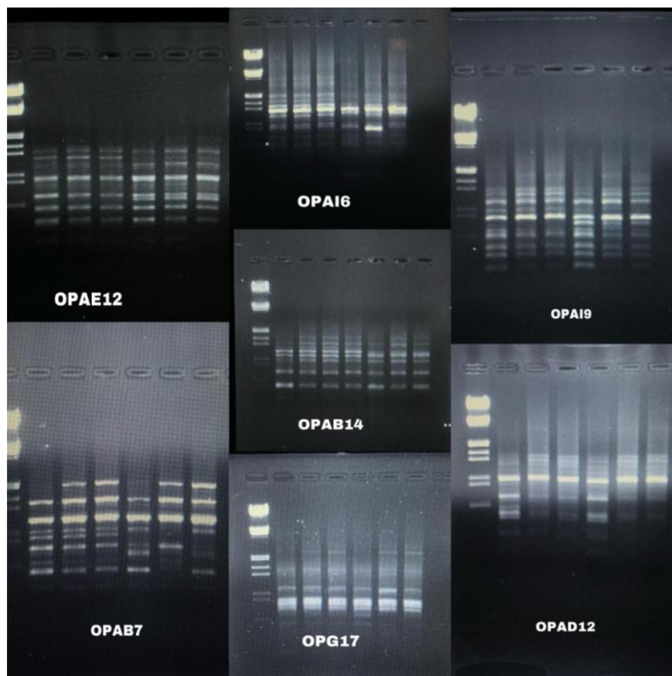

Fig. 1: Gel images of RAPD gels of selected primers by Geldoc – Vilber.

ISOLATE	CLONE	Lesion size after 96 hours (cms)																AVERAGE (cms)
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	
C1	RRII105	10	10	9	9	9	10	10	12	11	11	12	12	10	9	9	10	10.1875
	PB260	10	10	10	11	11	11	11	12	11	11	10	10	9	0	6	10	9.5625
C2	RRII105	10	10	10	9	9	10	9	9	10	9	9	10	10	10	9	9	9.5
	PB260	11	11	9	11	11	11	9	10	10	10	10	9	9	10	10	10	10.0625
C3	RRII105	10	11	12	12	12	11	11	12	12	11	11	12	12	13	12	11	11.5625
	PB260	10	12	11	10	10	10	9	9	9	10	10	10	10	9	9	9	9.8125
C4	RRII105	14	14	14	11	12	14	12	10	14	14	13	15	13	12	11	11	12.75
	PB260	12	11	11	11	10	12	12	12	12	12	12	11	11	12	12	12	11.5625
C5	RRII105	10	10	10	10	9	10	10	11	10	10	9	11	11	10	11	11	10.1875
	PB260	10	10	10	11	10	11	10	11	11	10	10	10	11	9	10	11	10.3125

Table 2.

CLONE	***Average Lesion Diameter (cms)***
RRII105	54.1875
PB260	51.3125

Table 3.

Fig 2: Lesions developed on RRII105 leaves after 96 hours incubation.

This difference aligns with the results of RAPD, as the difference in genetic composition is the very reason of changes in disease resistances. However, it's important to consider that the presence or absence of loci in both RAPD products may not be a reason for the change in disease resistances and the difference in lesion diameters may be not caused by the disease resistance, but because of the difference in the virulence mechanism of the fungal isolates, because virulence variation among fungal isolates may also influence the observed differences in lesions (Pereira et al., 2020). Therefore, future studies should employ standardized virulent strains to isolate host-based resistance more precisely. But still, it is clearly found that RRII105 and PB260 clones of *H. brasiliensis* has genetic dissimilarities with different DNA profiles and the clone *Hevea brasiliensis* RRII105 is slightly more resistant to *Corynespora cassiicola* Leaf Fall Disease because of the change in lesion diameters.

4. INTERPRETATIONS

The combined molecular and phenotypic results highlight the existence of genetic diversity between RRII105 and PB260 clones. RAPD markers confirmed that the two clones, though belonging to the same species, exhibit variations at the DNA level. These variations can be linked to slight differences in disease resistance, as evidenced by lesion size in virulence tests.

Although the magnitude of both genetic and phenotypic differences was modest, the findings emphasize the importance of integrating molecular tools with classical plant pathology assessments. Such integrated approaches are instrumental in clone certification, conservation, and resistance breeding in *Hevea brasiliensis* cultivation.

5. CONCLUSION

The present investigation successfully demonstrated that genetic variation exists between *Hevea brasiliensis* clones RRII105 and PB260, as evidenced by RAPD marker analysis and virulence testing against *Corynespora cassiicola*. The identification of polymorphic loci and the differential response to fungal infection suggest that these clones possess distinct genetic compositions that influence their resistance capacities. While PB260 showed a marginally higher resistance to *C. cassiicola*, the findings highlight the importance of minor genomic variations in shaping disease susceptibility and adaptability. This study underscores the value of using RAPD markers as a cost-effective molecular tool for early screening and differentiation of clones in breeding programs. Integrating such molecular methods with traditional plant pathology offers a more complete understanding of plant behavior, allowing for informed clone selection and improved resistance management.

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