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REVIEW ARTICLE

An attempt to validate molecular and field level screening results for the Corynospora leaf fall disease in rubber (*Hevea brasiliensis*)

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Abstract

Corynespora Leaf Fall (CLF) disease is one of the serious diseases, caused by Corynespora cassiicola, affecting rubber (Hevea brasiliensis L) plantations. Clones rated as resistant to the disease under the polybag nurseries became susceptible at the field level causing major problems in clone recommendations. Therefore, it is of utmost importance to add new CLF resistant genotypes to the breeding pool. Therefore, the present study was carried out to attempt to validate the molecular screening by field screening results. The molecular screening was carried out using 35 genotypes from 2005 hand-pollinated progeny, their grandparents (RRIC 100 and RRIC 103), grate grandparents (RRIC 52 and PB 86), and two check clones (RRISL 201 and RRISL 208). The 2005 hand-pollinated progeny which has comprised with self progenies, raised at 1978 hand pollination by selfing at CLF susceptible clone RRIC 103 and CLF resistant clone RRIC 100. Four SSR Primers (HB 1, HB 11, HB 29, hmct 5) were selected based on polymorphism between the CLF free clone RRIC 100 and susceptible clone RRIC 103 for molecular screening. Field screening was done at polybag nursery, budwood nursery, and at field level in three locations viz., Nivithigalakale, Monaragala, and Gallewatta. Completely randomized design (CRD) was used with five to ten replicates. Disease assessment was carried out allowing plants for the natural infection based on the index developed for scoring of disease severity. Observations were taken three times during peak and off seasons of CLF disease occurrence and were assessed along with control clones. All primers generated two fragments for Hevea and built the genetic distance matrix using a power maker (V 3.0) computer program and a tree diagram was drawn using the Tree view computer program. Cluster analyses revealed four distinct clusters. Two primary clones, PB 86 and RRIC 52, and the clones RRIC 103 and RRIC 201 were grouped and another cluster was again grouped into three main sub-clusters. Around 40% of field screening results obtained agreed with molecular grouping whereas, 57% were not agreed and around 3% of genotypes did not show a clear correlation. However, further screening at the field level and molecular screening is needed.

Key words: Corynespora leaf fall disease, Hevea breeding, molecular markers, resistance, SSR

Introduction

Natural rubber from *Hevea brasiliensis* is one of the most versatile industrial raw materials. Corynespora leaf fall (CLF) disease caused by *Corynespora cassiicola*, is a serious disease affecting rubber yield with relatively a recent origin. The disease now has become a serious threat to the natural rubber industry affecting several outstanding clones in Sri Lanka as well as in South and South East Asia, and Central Africa (Jayasinghe, 2000).

The clones identified as highly susceptible in Sri Lanka during the first epidemic in 1985-1986 are RRIC 103, RRIC 104, RRIM 600, Tjir 1, RRIM 725, IAN 873, and FX 25 (Jayasinghe and Silva, 1996). The use of chemicals to control CLF disease in mature fields is not an economically feasible method and also creates environmental and health hazards where it is not recommended by the Rubber Research Institute (RRI) of Sri Lanka, at present.

The sudden susceptibility of the resistant high-yielding clones, which came through laborious evaluation procedures, has raised a big problem for rubber breeders and makes it difficult to recommend a clone for growers with confidence. Clones which showed resistance to Corynespora leaf fall during the evaluation were susceptible at field level after some time and disturb the clone recommendations. Therefore, it is very important to add precise Corynespora leaf fall-resistant genotypes to the breeding pool. The development of Corynespora leaf fall-resistant clones along with other performances such as high yield and vigor is a great challenge in *Hevea* breeding. The laboratory-based *in vitro* screening methods are not dependable and *in vitro* screening methods should be used only to obtain preliminary data. This also consumes considerable time under field experiments. And also field evaluation through visual observation and laboratory assays through excised leaf inoculation have led to the screening of putatively resistant Wickham clones and wild germplasm. However, laboratory bioassays are preliminary and may not ensure actual field-level resistance as observed for the disease of other forest trees. Many man-hours of labor and enormous quantities of fungicidal chemicals have been reported to be required every year for the management of the above disease in a vast area of rubber plantations in India and other rubber growing countries. The cost of fungicides and their long-term effect on the environment justify the need for breeding disease-resistant trees.

Molecular markers have now been proved very useful in selecting disease resistant clones (Collard *et. al.,* 2004). SSR (Simple Sequence Repeats) is a newly selected molecular marker to detect the resistant gene of different plant species due to its numerous advantages like hypervariability, displaying high levels of polymorphism, and ease of detection by PCR (Mantello *et al.*, 2012). Four SSR Primers (HB 1, HB 11, HB 29, *hmct 5*) were selected based on polymorphism between the CLF disease free clone RRIC 100 and the complete susceptible clone RRIC 103 (Tharanga *et*

al., 2018). Hand pollinated progeny developed in the year 2005 showed a wide range of CLF disease responses, as complete susceptible to free from the disease, is having second self progenies of the clone RRIC 100 as well as the clone RRIC 103. 2005 hand pollinated progeny which has comprised with self progenies, was raised at 1978 hand pollination by selfing at CLF susceptible clone RRIC 103 and CLF resistant clone RRIC 100. The resistance screening using SSR marker to select high performing CLF resistant rubber clones and correlation between molecular and field screenings have not yet been undertaken. Therefore, the current study was carried out with the objective of the attempt to validating the results of Microsatellite based molecular markers during molecular screening for CLF resistance with field level screening for the disease resistance.

Methodology

The experiment was carried out at the Department of Genetics and Plant breeding of Rubber Research Institute, Nivithigalakale substation, Matugama, Monaragala Substations, and Gallewatta estate.

Thirty five genotypes from the 2005 hand pollinated progeny program, with their grandparents (RRIC 100 and RRIC 103), great grandparents (RRIC 52 and PB 86) and with two check clones as RRISL 201 and RRISL, 208 were selected for the study.

Thirty five genotypes from the 2005 hand pollinated progeny which has comprised with self progenies of three *Hevea* genotypes that 1978 HP 375, 1978 HP 377 and 1978 HP 878 raised at 1978 hand pollination. That three 1978 HP genotypes already CLF resistant. The 1978 HP 375 and 1978 HP 377 were raised by selfing at CLF susceptible clone RRIC 103. 1978 HP 878 was raised by selfing with CLF resistant clone RRIC 100.

Molecular screening

DNA extraction

The genomic DNA extraction from leaves at the immature apple green stage was done according to the mini preparation method developed at RRISL (Withanage, 2013). The Purity and the concentration of extracted DNA samples were checked using agarose gel electrophoresis and visualized under gel documentation. Initially, thirty SSR primers were supplied by AVON PHARMO CHEM Private Ltd., USA, (*i.e.* HB1 to HB4, HB6 to HB12, HB14 to HB22, HB24 to HB30, *hmct* 5, *hmac*4, and *hmtc*1) were used.

PCR amplification

PCR optimization was performed with minor changes with a standard protocol developed at RRISL to obtain clear and precise repeatable fragments. PCR amplification was done in 20 µl reaction volume containing 50-100 ng template DNA with 1× PCR buffer, 2mM dNTPs, and one unit of Taq polymerase (Gene Tech, Sri Lanka) and 5mM of primer. Amplification was performed in Multigene DNA thermal cycler (Multi gene, Lab Net international Inc.) and the program consists of an initial denaturing

step at 94 ºC for, 4 minutes, 35 cycles of 1 minute at 94 ºC, 1 minute at the specific annealing temperature of each primer pair, and 2 min at 72 ^{0}C, followed by a final extension reaction. The amplified PCR products were resolved in 1.5% Agarose containing gel. The banding pattern was visualized in gel documentation.

Data analysis

Power marker software program, version 3.0 (Liu, 2004) was used to develop a phylogenetic tree and genetic distance matrix for the analysis. The construction of the phylogenetic tree was based on the Unweighted Pair Group Method (Koichiro Tamura *et al.*, 2013) with Arithmetic Averages (UPGMA) embedded in the MEGA6 software.

Field screening

All experimental materials, thirty five genotypes from 2005 hand pollination progeny, with their grandparents (RRIC 100 and RRIC 103), great grandparents (RRIC 52 and PB 86), and with check clone as RRISL 201 used were established at the RRISL substations in Nivithigalakelle, Gallewaththa estate (a traditional rubber growing area) and Monaragala (a non-traditional rubber growing area).

Screening of genotypes and their control clones in polybag and budwood nurseries at Nivithigalakele and in field establishments at Galewatta estate and Monaragala Substation

A completely randomized design (CRD) was used with five to ten replicates per genotype. Plants were screened for CLF disease resistance under natural infection and three observations were taken in the one-year-old plants. Five to ten plants were demarcated for the observations and the disease severity assessment was carried out based on the index for scoring of disease severity (score index) as shown in Table1. The results of the field experiment mean score index were subjected to cluster analysis to distinguish resistance of CLFD.

Table 1. *Score index for the assessment of CLF disease severity*

Index for scoring of disease severity (score index)	Description
0	No disease
1	Mild (0-25% of the leaf area is covered by the disease)
2	Moderate (25-50% of the leaf area is covered by the disease)
3	Severe (50-75% of the leaf area is covered by the disease)
4	Very severe (> 75% of the leaf area is covered by the disease)

(Fernando *et al.*, 2010)

Results and Discussion

In molecular screening, a pairwise genetic distance matrix was developed, based on two amplified DNA fragments of four primers, using Power marker program V 3.25. According to the dendrogram constructed using the "MEGA 6.06" computer program (V 3.25), two primary clones PB 86 and RRIC 52, and also the clones RRIC 103 and RRIC 201 were grouped together and the other cluster was again grouped into three sub-clusters. When considered the clustering pattern along with their pedigree, it is showed their close genetic relationship, by grouping genotypes that shared the same parentage (Fig.1a and 1b).

All thirty five genotypes of 2005 hand pollination progeny were grouped into the second main cluster named B, which was again divided into three clusters as B1, B2, and B3 (Fig. 1b). Cluster B1 comprised of the clone RRISL 208 and few genotypes. Cluster B2 was comprised of 20 genotypes and cluster B3 was grouped with the clone RRIC 100. The CLF moderately susceptible clone RRISL208 was grouped with HP 09, HP 05, HP 03, and HP 04 (group B1) and those genotypes were the products of CLF resistant genotype 1978 HP which showed moderately susceptible CLF response at field screening. The cluster B2 comprised with genotypes HP 1, HP 11, HP 13, HP 19, HP 23, HP 32, HP 58, HP 26, HP 10, HP 46, HP 50, HP 52, HP 21, HP 22, HP 06, HP 07, HP 15, HP 17, HP 2 and HP 49.

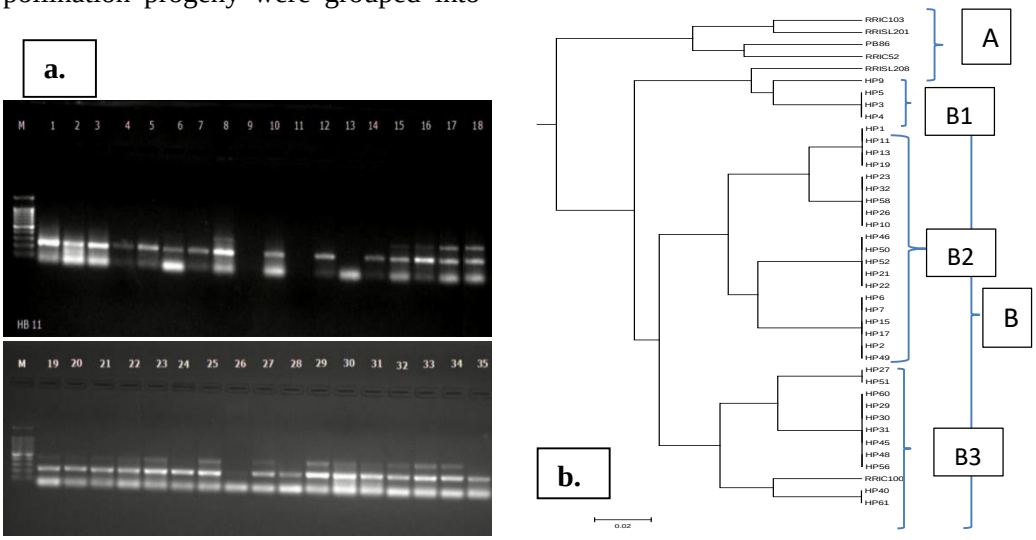

Fig. 1. Cluster analysis of the molecular screening and field screening to identify CLFD resistance **a.** Microsatellite profile of HB 11 primer for 35 genotypes in 2005 Hand pollination progeny **b.** Dendrogram of recommended clones including grand grandparents, grandparents of 2005 Hand pollination progeny, moderate susceptible recommend clones and 35 genotypes of 2005 hand pollination progeny in molecular screening

Variation between genotypes and clones was observed, however, it is unable to explain this situation clearly by looking at the relationship between field screening and molecular variation.
Therefore, further studies are needed to carry out to confirm the molecular relationship for the field level, CLF disease response in *Hevea*.

Field screening

In Nivithigalakale polybag screening, cluster number one consisted of ten genotypes along with disease free clones RRIC 100 and PB 86. Out of these 10 genotypes, two genotypes (2005 HP 5 and 2005 HP 1) were free from the disease while the rest of the eight genotypes showed very low disease intensity. The second cluster was grouped with 15 genotypes which were moderately susceptible to the CLF disease along with susceptible clone RRIC 103 and moderately susceptible clone RRISL 201. The third cluster was comprised of nine susceptible genotypes along with susceptible clone RRIC 52.
In Nivithigalakale, budwood nursery screening, cluster number one consisted of seven genotypes, along with moderately susceptible clone RRISL 201 and severely susceptible clone RRIC 103. The second cluster had 14 genotypes with disease free resistance clone RRIC 100 and resistance clone PB 86. The third cluster comprised the susceptible clone RRIC 52 and 10 other genotypes.
Cluster number one was grouped with nineteen genotypes along with disease free clones RRIC 100 and PB 86 at Monaragala during the field screening where two genotypes (2005 HP1 and 2005 HP2) were free from the disease while the rest of 19 genotypes showed very low disease severity. The second cluster had 10 genotypes with the moderately susceptible clone RRISL 201. The third cluster comprised of the susceptible clones RRIC 52 and RRIC 103, and six genotypes.
In the field screening at Galewatta, cluster number one consisted of 24 genotypes along with two CLF disease resistant clones RRIC 100 and PB 86. Out of those, 2005 HP1 and 2005 HP3 were free from the disease and the rest of the genotypes were also showed very low disease severity (Mean score 213). The second cluster had five genotypes and the moderate susceptible clone, RRISL 201. The third cluster compared with the susceptible clones RRIC 52 and RRIC 103 and four other genotypes.
Fernando *et al.* (2010) found the different methods to evaluate the susceptibility and resistance of genotype screening methods and are not dependable and should be used only to obtain preliminary data.
The studies of Manju and coworkers (2010) showed a differential behavior for CLF disease infection in the field and nursery experiments. It would be worthwhile to look for genes conferring resistance in the first cluster as its genetic base is much wider than the remaining two clusters. The continuous distributions of resistance patterns within a population of 62 clones suggest involvement of quantitative inheritance

to the resistance of *Corynespora cassicola*.

A comprehensive review of the clonal susceptibility to CLF disease in various rubber growing countries (Mathew, 2006) suggested that the tolerance level of widely cultivated clones is declining and also the creditability of the resistance level of a cultivated clone is lost due to breaking down of previously known resistance. According to the findings of Othman *et al.* (1996), the clone RRIM 600 and GT1 earlier reported susceptible as a result of the development of newer races of pathogen favored by the exposure to a long period of monoculture.

Verification of molecular screening results by field screening

When, developing a relationship between field screening and molecular screening, around 40% [(14/35)*100] of the genotypes screened were agreed in both field level response and molecular grouping and around 57% [(20/35)*100 did not show similar results. Around 3% [(1/35)*100 showed varied results and could not be concluded (Table 2).

In general, *Hevea* clones and genotypes studied in this experiment showed a differential behavior for CLF disease infection in the field, nursery, and molecular screening. It would be worthwhile to look for genes conferring resistance in the first cluster as its genetic constituents distributions resistance pattern within a population.

According to dendrogram, molecular screening was grouped resistant, susceptible and moderate susceptible for control clones. As when grouped with RRIC 100 it has assumed having resistance), groped with RRISL 208 (assumed moderate susceptible) and grouped without RRIC 100 and RRISL 208 (assume susceptible) in their nursery and field screening (Table 2).

Table 2. *Verification of field screening results through molecular screening*

Confirmed the field screening by the molecular result	Unconfirmed the field screening by the molecular result	Vary in result with molecular results
05 HP 27	2005 HP 29	2005 HP 48
2005 HP 30	2005 HP 31	
2005 HP 40	2005 HP 56	
2005 HP 45	2005 HP 61	
2005 HP 51	2005 HP 1	
2005 HP 60	2005 HP 2	
2005 HP 4	2005 HP 10	
2005 HP 5	2005 HP 11	
2005 HP 9	2005 HP 13	
2005 HP 6	2005 HP 17	
2005 HP 7	2005 HP 19	

Confirmed the field screening by the molecular result	Unconfirmed the field screening by the molecular result	Vary in result with molecular results
2005 HP 15	2005 HP 21	
2005 HP 46	2005 HP 22	
2005 HP 3	2005 HP 23	
	2005 HP 26	
	2005 HP 32	
	2005 HP 49	
	2005 HP 50	
	2005 HP 52	
	2005 HP 58	

[The results were obtained based on cluster groups (resistance, moderate resistance, and susceptibility) produced in molecular screening at the poly bag and budwood nurseries and field establishments at Galewatta estate and Monaragala substation]

Conclusion

The 2005 hand pollination progeny showed a range of CLF disease responses *i.e*. free from the disease to severe susceptibility.

Hevea clones and genotypes studied showed a differential behavior for CLF disease infection in the field, nursery, and molecular screening. Forty percent of the studied genotypes confirmed the molecular grouping by field screening and around 57% of genotypes did not develop the correlation. Around 3% of genotypes did not produce a clear relationship. It would be worthwhile to look for genes conferring resistance to CLF in the first cluster as its genetics continuous resistance distribution pattern within a population.

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