Screening Susceptibility of Hevea *Progenies from PB 5/51 X IAN 873 to Two Races of* Corynespora cassiicola

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Two races of Corynespora cassiicola *have been found to affect immature and mature leaves of* Hevea brasiliensis *in Malaysia. Immature and mature leaves of 20 randomly selected progenies from family PB 5/51 X IAN 873 were used to screen resistance/susceptibility to isolates CSB 16 (race 1) and CLN 16 (race 2) separately. Analysis of Variance (ANOVA) and the Least Significant Difference (LSD) test at a 0.05 probability level were used to compare treatment means. The multifactor ANOVA was used to analyse the results which include main effects and interactions between factors studied viz. progenies, leaf types and isolates. Immature leaves were more susceptible to both races than mature leaves and isolate CLN 16 (race 2) was the more virulent to this* Hevea *family. This study was able to detect five progenies that were resistant to both fungal isolates. They were progenies 1636, 1747, 3223, 3320 and 3562.*

Keywords: *Corynespora cassiicola*; leaf types; isolate; races; disease severity

The *Corynespora* leaf fall (CLF) disease is caused by *Corynespora cassiicola* [(Berk. & Curt) Wei]. It was first detected in Malaysia in 1960[1]. It was a minor leaf disease then as it attacked certain clones in the budwood nurseries[2], but presently there is an increased incidence of the disease in Malaysia.

Corynespora leaf fall in Malaysia may not be as important a disease to rubber like the South American Leaf Blight (SALB), but if left unchecked it may be so with the estimated crop loss due to CLF amounting 20% in 1990[3], 25% in 2007[4] and recently up to 40% in 2009[5].

The economic loss due to this disease can be more serious with the discovery of different races of *C. cassiicola*. Ismail and Jeyanayagi[6] discovered that two different races exist within *C. cassiicola* and classified them race 1 and race 2. Race 1 of the pathogen was found to infect the earlier *Hevea* clones *e.g.* RRIM 600 while race 2 infects the newer clones *e.g.* RRIM 2020. Nghia *et al.*[7] recently discovered the possible emergence of race 3 of this fungus. A major disease outbreak can be feared, as many rubber tree clones initially described as resistant to the disease have become susceptible, suggesting a good adaptability of the pathogen[4,8].

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The severity of the disease is influenced in part by the susceptibility of the rubber clones. In a susceptible clone, infection with *C. cassiicola* will result in complete defoliation and the affected tree becomes stunted. Previous methods of controlling the disease were to avoid planting susceptible clones and *via* chemical control[9]. A practical way to combat the disease is early detection of the susceptible clones.

In the past, differentiation of *C. cassiicola* isolates was made visually[9–14]. With the advent of molecular marker techniques, they have become potential tools for understanding genetic diversity and epidemiology of fungal pathogens is available[15]. In a preliminary study, Chow and Low[16] found polymorphism between the 15 isolates with the use of random amplified polymorphic DNA (RAPD) molecular marker technique. Furthermore, two races of the fungus were distinguished using the RAPD technique, internal transcribed spacers (ITS) markers[17,18] and *via* analysis using the inter simple sequence repeats (ISSR) markers[7].

This paper will discuss the screening of progenies from family PB 5/51 X IAN 873 against race 1 (isolate CSB 16) and race 2 (isolate CLN 16) of *C. cassiicola*, the interaction between the two different races against the different leaf types (immature and mature) of *Hevea* and to identify the progenies that are resistant to both isolates using statistical analysis.

MATERIALS AND METHODS

Plant Materials

Leaves at two different developmental stages *i.e.* just hardened/light green (stage C) and mature/dark green leaves (stage D) (*Figure 1*), were sampled from 20 randomly chosen progenies from a population of 137 (14.5%) from family PB 5/51 X IAN 873. Clone PB 5/51 is an Oriental clone with superior production of latex and one of the most popular clones used in breeding programmes in Malaysia. IAN 873 is a Brazilian clone that was brought to Malaysia in 1951 and was found to have a superior bole height and wood density but is susceptible to *C. cassiicola*[19]. As control, leaves from clones RRIM 600 and RRIM 2020 were used.

Source of Isolates

Two *C. cassiicola* isolates were used in this study: CSB 16 (race 1) collected from Sg. Buloh, Selangor (*Figure 2*) and CLN 16 (race 2) collected from Lanchang, Negeri Sembilan (*Figure 3*). Both isolates were distinctive from each other. CSB 16 culture produced a dark mycelial mat and its conidia were small, long and thin. Meanwhile, CLN 16 culture was light brown in colour and produced shorter and wider conidia. Isolate CSB 16 has been reported to infect older *Hevea* clones such as RRIM 600, whilst isolate CLN 16 infects newer Latex Timber Clone (LTC) *i.e.* clone RRIM 2020[6].

Fungal Isolation

The lesions obtained from infected leaves were cut in half and were sterilised with alcoholic mercuric chloride solution (0.1% mercuric chloride, 75% ethanol) for 1 min, rinsed in distilled water (4–5 times) and plated in Petri plates containing 15 mL of sterile Potato Sucrose Agar (PSA). The plates were incubated at room temperature (28 ± 2ºC) in darkness for five days. Pure cultures were kept in the Crop Improvement and Protection Unit (CIPU), Malaysian Rubber Board (MRB) fungal bank.
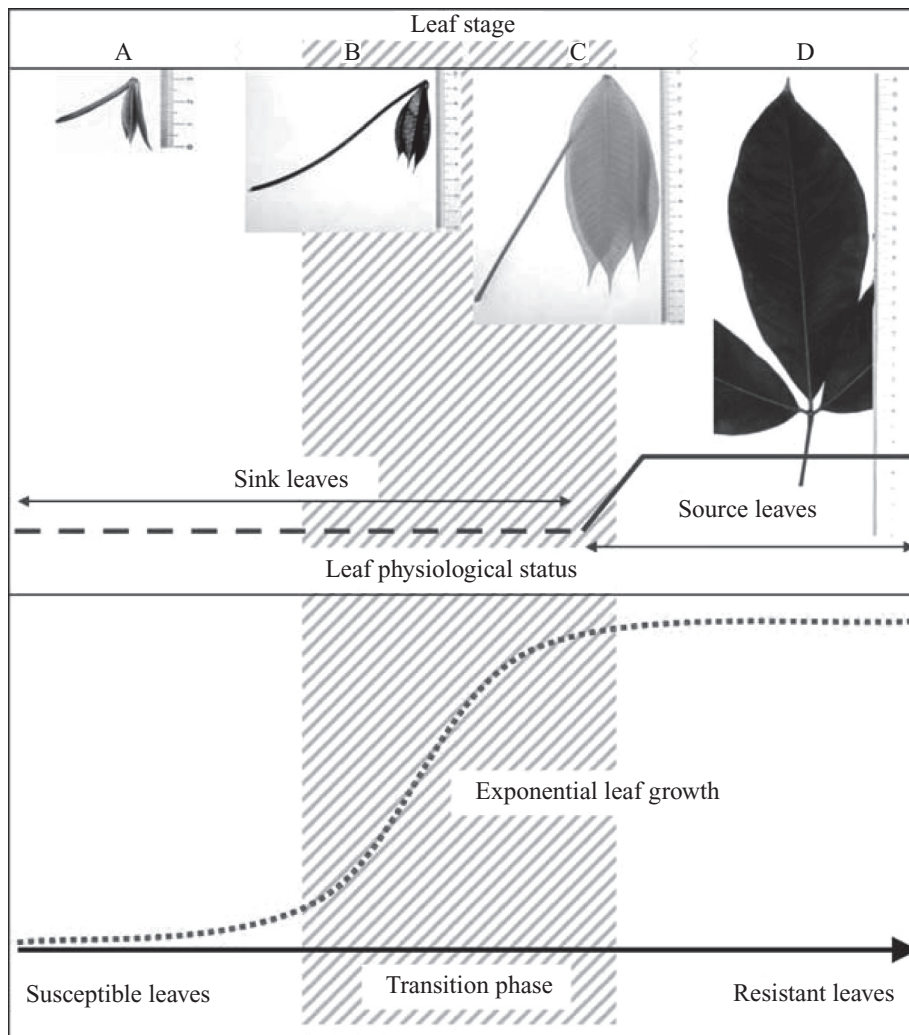
Figure 1. Relationship between exponential leaf growth, transition phase from completely susceptible leaf stage to completely resistant leaf stage, and the short physiological step from sink to source leaves. This qualitative comparison attempts to visualise the different developmental processes that turn a susceptible rubber leaf into a completely age-resistant organ[20].

Preparation of Inoculum

Pure cultures grown on PSA in Petri plates were grown in the dark for five days and then exposed to flourescent light for another five days to stimulate sporulation. Conidia suspension was prepared by flooding the Petri plate with 50 mL of sterile distilled water and scraping the mycelium mat gently with a sterile glass rod. The spore suspension was adjusted to a concentration of 2×10^3 spores per mL.
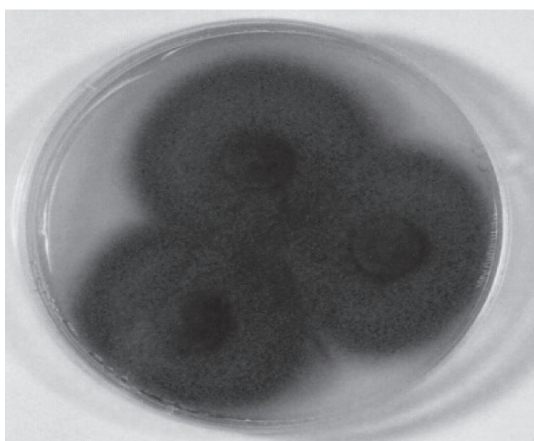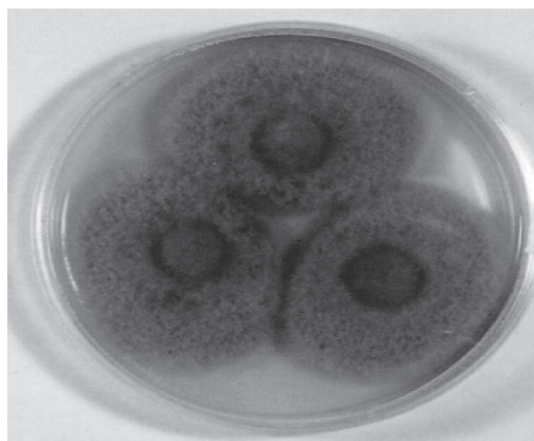
Figure 2. Morphology of CSB 16 isolate.

Figure 3. Morphology of CLN 16 isolate.

Leaf Disc Preparation and Inoculation

The method used was as described by Chee[9] with some modifications. Immature (just hardened, light green) and mature (hardened, dark green) leaves were collected from 20 randomly selected progenies of family PB 5/51 X IAN 873. The leaves were rinsed three times in sterile distilled water to ensure the leaves surfaces were clean of debris, dust and fungicide. Leaf discs of 15 mm in diameter were cut out from each progeny using a cork borer and the discs were floated with abaxial surface facing upwards in a Petri plate containing $20 - 25$ mL sterile distilled water. Conidial suspension containing 2×10^3 spores per mL prepared from a 10 day old culture of *C. cassiicola* isolates was sprayed using a hand atomizer (Sigma®) onto the leaf discs. The Petri plates containing the leaf discs were incubated under flourescent light at $24 - 25$ºC for a week. The severity of infection was scored on a scale of 0 (devoid of any infection) to 5 (severe infection) based on the quantity of lesions (*Figure 4*; *Table 1*).

Experimental Design and Data Analysis

The three factors studied in this experiment were progenies, leaf types and isolates.

The experimental design was a randomised complete block design (RCBD) in which each Petri plate contained five leaf discs (15 mm each) and five Petri plates per *Hevea* progeny. The experiments were run by blocking the repetitions (the experiment was repeated twice, each time on a different day) and randomly selecting the progenies, leaf types and isolates (*Figure 5*).

Infection was scored qualitatively based on morphological observation with reference to a disease score chart with scores of $0 - 5$ (*Figure 4*; *Table 1*). The Disease Severity Percentages for each progeny were calculated by averaging the scores for five leaf discs per replicate for each progeny. The data were analysed using Statistical Analysis System (SAS) software ver. 9 for Analysis of Variance (ANOVA) using the PROC GLM function and the Least Significant Difference (LSD) test at
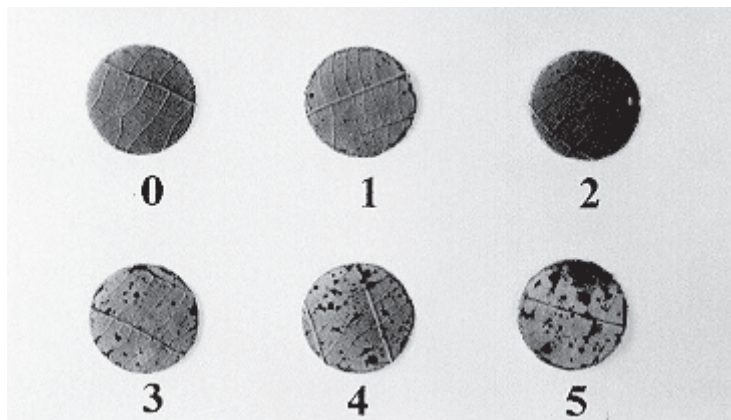
Figure 4. Disease score chart for Corynespora *Leaf Disease.*

TABLE 1. THE SEVERITY SCALE OF *CORYNESPORA* LEAF SPOT DISEASE SYMPTOM[21]

Score	Quantity of lesions covering the leaf discs (%)
0	0
1	1 - 10
2	11 - 25
3	26 - 50
4	51 - 75
5	>75

the 0.05 probability level is used to compare treatment means (*Table 2*).

The multifactor ANOVA was used to analyse the results which include main effects and interactions between factors studied *viz.* progenies, leaf types and isolates.

RESULTS AND DISCUSSION

Comparisons between Progenies, Leaf Types and Fungal Isolates

The susceptibility of rubber clones to *C. cassiicola* is dependant on the isolates and the rubber clone, a clone which is susceptible to an isolate may be resistant to another isolate[20–23]. Resistance to CLF is complex as it may be governed either by polygenic and/or monogenic inheritance[8]. In addition, the major genetic variation of the *Corynespora* disease resistance could be attributed to additive gene as well as non additive gene control from a study using five-parent diallel cross[24].

When the disease severity (DS) percentages were plotted against days of infection, generally CLN 16 (race 2) was observed to be more virulent than CSB 16 (race 1) (*Figure 6*). Isolate CLN 16 has a higher percentage of DS (21%) on day 2 after inoculation as compared
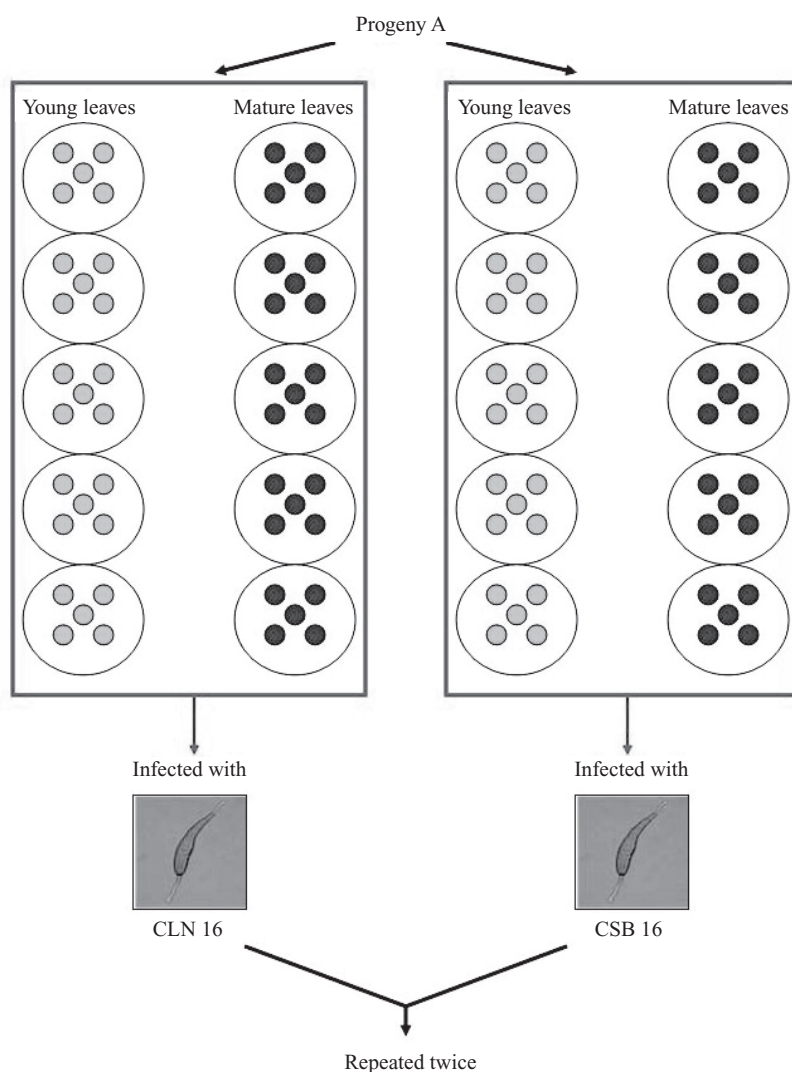
Figure 5. Schematic representation of the disease screening exercise.
The leaves from each progeny were similarly screened.

to isolate CSB 16 (11%). This suggests that the isolate CLN 16 is more virulent to this *Hevea* family than isolate CSB 16.

Young, just hardened/immature leaves were observed to be more susceptible to the fungus (*Figure 7*) than the mature leaves. Disease severity percentage for young leaves was 20%

whereas DS percentage on mature leaves was about 10% on day two after inoculation.

When progenies, leaf types and isolates were analysed, significant differences among the progenies were observed starting from day four. The susceptibility of immature and mature leaves was significantly different from
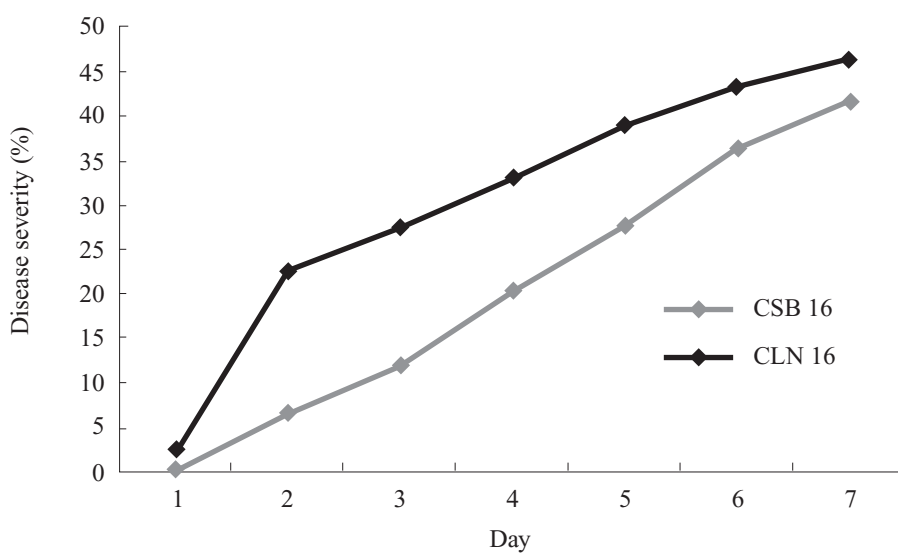
Figure 6. Disease severity (%) of Hevea *progenies to two* C. cassiicola *isolates.*
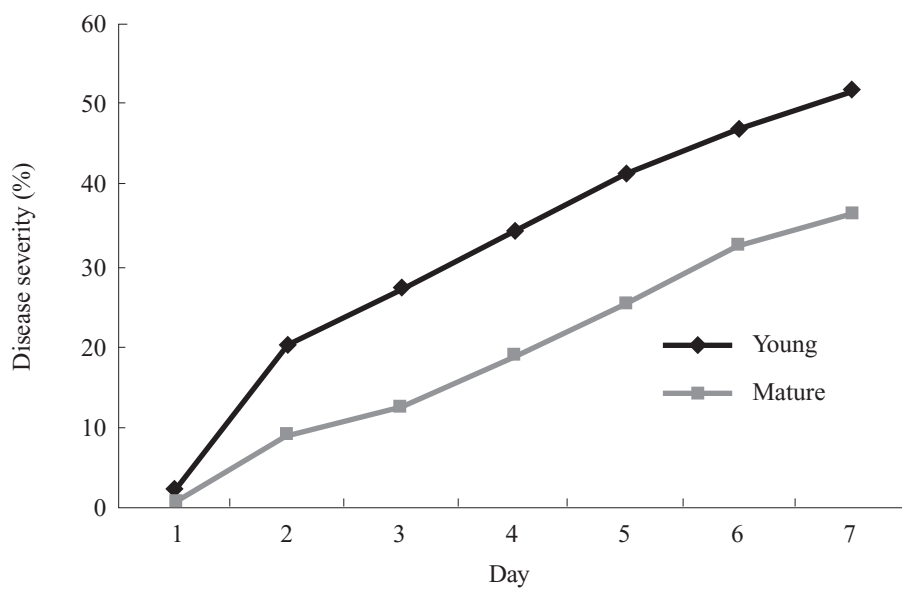

Figure 7. Disease severity (%) at different days after inoculation of two leaf types.

TABLE 2. RESULTS OF TWO WAY ANALYSIS OF VARIANCE OF INFECTION RATE OVER TIME (DAY)

Source of Variation	df	Days												
		1		2		3		4		5		6		7	
		MS	VR	MS	VR	MS	VR	MS	VR	MS	VR	MS	VR	MS	VR
Reps	1	2.08	16.21***	1.27	12.15***	0.65	6.01**	0.72	7.57***	0.49	4.96**	0.59	6.63**	0.88	8.76***
Progenies	21	0.38	2.98***	0.16	1.53ns	0.21	1.91**	0.26	2.72***	0.31	3.17***	0.43	4.82***	0.52	5.21***
Leaf types	1	1.76	13.73***	2.56	24.52***	3.08	28.41***	3.15	33.13***	3.06	30.75***	1.97	22.01***	1.68	16.73***
Isolate	1	5.37	41.90***	14.30	136.78***	15.46	142.20***	10.82	113.64***	7.46	75.06***	5.51	61.46***	4.12	41.08***
Progenies* Leaf types	21	0.05	0.38ns	0.05	0.51ns	0.05	0.50ns	0.06	0.66ns	0.06	0.59ns	0.04	0.46ns	0.04	0.40ns
Progenies* Isolate	19	0.14	1.07ns	0.23	2.23ns	0.37	3.38***	0.44	4.58***	0.49	4.88***	0.58	6.47***	0.61	6.07***
Leaf types* Isolate	1	0.38	3.00ns	0.79	7.57ns	0.15	1.36ns	0.02	0.25ns	0.00	0.00ns	0.01	0.09ns	0.01	0.09ns
Progenies* Leaf types* Isolate	19	0.03	0.22ns	0.02	0.21ns	0.05	0.46ns	0.05	0.57ns	0.05	0.47ns	0.03	0.31ns	0.02	0.19ns

*(p<0.05), ** (p<0.01), *** (p<0.001), ns (p>0.05), MS = Means square; VR = Variance ratio

each other and the immature leaves were more susceptible to infection with either CSB 16 or CLN 16 (*Table 3*). This statistical observation mirrors the results of the plotted percentage of disease severity against days of observations (*Figure 7*).

There was no significant interaction between progenies and leaf types and between leaf types and isolates from day one to day seven for the mean of infection rate at the 0.05 probability level. This means that both leaf types (immature and mature leaves) of each progeny are similar to each other and the effect of both fungal isolates on the two different leaf types of every progeny are similar. On the other hand, there was a significant interaction between progenies and isolates for mean of infection rate starting from day three indicating that clonal differences among the progenies has begun to emerge.

It is well documented that the fungus *C. cassicola* infects *Hevea* leaves at all stages (immature and mature)[8,25] though no statistical proof was ever produced. This study has proven statistically that immature leaves are more susceptible to fungal infection than

mature leaves. The immature leaves from a sample of this family (PB 5/51 X IAN 873) were more susceptible to infection from both isolates CSB 16 and CLN 16 and perhaps to other *C. cassiicola* isolates. This could be due to the fact that immature leaves have yet to develop the lignin needed as an external defense against the infection of the fungus.

Lieberei[20] discussed in length the physiological changes in rubber leaf properties that occur from bud burst until hardening of the leaves. Leaf development stages are divided into groups A to D (*Figure 1*). Leaf stages A, B and C are thin, devoid of any resistance against virulent isolates and almost free of lignin. The presence of lignin was detected in leaf structure of stage D which coincides with the onset of leaf hardening. At this stage the leaf has become somewhat resistant against virulent isolates.

In regards to the interaction between the fungal isolates, CSB 16 and CLN 16 were significantly different from each other (*Table 4*) thus strengthens the proof that isolate CLN 16 (race 2) was more virulent to this family (PB 5/51 X IAN 873) than isolate CSB 16 (race 1).

TABLE 3. RESULTS OF THE LEAST SIGNIFICANT DIFFERENCE TEST FOR MEAN INFECTION RATE OF LEAF TYPE AT 7 DAYS AFTER INOCULATION

Leaves comparison	Difference between means
Immature – mature	0.197***

LSD = 0.0972, t = 1.988, α = 0.05, df_e = 83, MSE =0.100

TABLE 4. RESULTS OF THE LEAST SIGNIFICANT DIFFERENCE TEST FOR MEAN INFECTION RATE OF FUNGAL ISOLATES AT 7 DAYS AFTER INOCULATION

Isolates comparison	Difference between means
CLN 16 – CSB 16	0.274***

LSD = 0.0972, t = 1.98, α = 0.05, df_e = 83, MSE =0.100

Comparisons Between Each Clone to RRIM 2020 or RRIM 600

Two clones, RRIM 2020 and RRIM 600, were used as control in the experiment. These two clones were used as control in screening for resistance/susceptibility to *C. cassiicola* in the laboratoty, as RRIM 2020 is susceptible to CLN 16 and RRIM 600 is susceptible to CSB 16.

Sixteen progenies (40% of the sampling) were not significantly different at $p \leq 0.05$ from RRIM 600 and 5 progenies (25% of the sampling) were significantly different at $p \leq 0.05$ from RRIM 2020 when the Least Significant Difference test for the mean comparison was conducted (*Tables 5a* and

5b). These progenies were clones 1636, 1747, 3223, 3320 and 3562.

Five out of 20 progenies (25%) were significantly different from RRIM 2020 at $p \leq 0.05$. This means that 75% of the progenies were "reasonably susceptible" or "susceptible" to CLN 16. On the other hand, 16 out of 20 progenies (80%) were significantly different at $p \leq 0.05$ from RRIM 600 which is used as a standard control in screening against isolate CSB 16. In other words, 80% of the progenies here "reasonably resistant" or "resistant" to CSB 16. The five progenies (1636, 1747, 3223, 3320 and 3562) that were resistant to CLN 16 were also resistant to CSB 16. This indicates that these progenies could be extremely resistant to the fungus in general.

TABLE 5A. RESULTS OF THE LEAST SIGNIFICANT DIFFERENCE TEST FOR MEAN INFECTION RATE OF PROGENIES AT 7 DAYS AFTER INOCULATION FOR RRIM 600

Progeny	Mean + s.e	Progeny Comparison	Difference between means
1324	3.149+0.626	1324-RRIM600	0.503ns
1432	0.823+0.276	1432-RRIM600	−2.068*
1548	0.202+0.039	1548-RRIM600	−3.633*
1636	1.159+0.377	1636-RRIM600	−1.813*
1741	0.953+0.251	1741-RRIM600	−2.563*
1747	0.801+0.201	1747-RRIM600	−2.943*
2573	1.543+0.438	2573-RRIM600	−1.523*
2872	2.710+0.710	2872-RRIM600	0.478ns
2975	0.828+0.117	2975-RRIM600	−3.033*
3029	0.944+0.191	3029-RRIM600	−2.717*
3070	0.856+0.201	3070-RRIM600	−2.853*
3072	2.840+0.648	3072-RRIM600	0.263ns
3168	2.404+0.533	3168-RRIM600	−0.708ns
3171	1.129+0.399	3171-RRIM600	−1.543*
3223	0.593+0.155	3223-RRIM600	−2.998*
3320	0.376+0.102	3320-RRIM600	−3.408*
3368	0.683+0.178	3368-RRIM600	−2.758*
3426	1.199+0.368	3426-RRIM600	−1.843*
3468	0.349+0.116	3468-RRIM600	−3.373*
3562	0.510+0.099	3562-RRIM600	−3.368*

LSD = 1.0878, t = 2.01808, α = 0.05, df_e = 42, MSE = 7.434

TABLE 5B. RESULTS OF THE LEAST SIGNIFICANT DIFFERENCE TEST FOR MEAN INFECTION RATE OF PROGENIES AT 7 DAYS AFTER INOCULATION FOR RRIM2020

Progeny	Mean + s.e	Progeny Comparison	Difference between means
1324	1.240+0.252	1324-RRIM2020	0.658ns
1432	1.750+0.319	1432-RRIM2020	0.528ns
1548	1.970+0.332	1548-RRIM2020	0.348ns
1636	2.930+0.451	1636-RRIM2020	2.018*
1741	2.570+0.383	1741-RRIM2020	1.313ns
1747	3.940+0.360	1747-RRIM2020	1.848*
2573	2.340+0.360	2573-RRIM2020	1.303ns
2872	1.120+0.325	2872-RRIM2020	−0.243ns
2975	1.880+0.297	2975-RRIM2020	0.518ns
3029	1.700+0.238	3029-RRIM2020	0.543ns
3070	1.650+0.194	3070-RRIM2020	0.268ns
3072	1.550+0.138	3072-RRIM2020	−0.193ns
3168	2.890+0.238	3168-RRIM2020	1.068ns
3171	2.420+0.404	3171-RRIM2020	1.358ns
3223	3.880+0.357	3223-RRIM2020	2.593*
3320	2.500+0.497	3320-RRIM2020	1.523*
3368	2.890+0.375	3368-RRIM2020	1.538*
3426	2.750+0.431	3426-RRIM2020	1.393ns
3468	1.620+0.266	3468-RRIM2020	0.528ns
3562	1.370+0.169	3562-RRIM2020	0.048ns

LSD = 1.4779, t = 2.01808, α = 0.05, df_e = 42, MSE = 1.073

CONCLUSIONS

This study proves that immature leaves are more susceptible to *C. cassiicola* than mature leaves. Isolate CLN 16 (race 2) was also proven to be more virulent to this *Hevea* family (PB 5/51 X IAN 873) than isolate CSB 16 (race 1).

Further more, this study was able to detect five progenies from PB 5/51 X IAN 873 were shown to be extremely resistant to both isolates and could be recommended for further screening in the field.

ACKNOWLEDGEMENTS

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