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Susceptibility of Rubber (*Hevea brasiliensis*) Clones to *Neofusicoccum ribis*

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Abstract The aim of this study was to evaluate the ability of *Neofusicoccum ribis* to infect leaf surfaces of different rubber (*Hevea brasiliensis*) clones. *Neofusicoccum ribis* isolates previously identified on the basis of morphological characteristics and DNA sequence analysis were used to inoculate rubber leaves and seedlings *in vitro* and *in vivo* respectively. *Neofusicoccum ribis* isolates were demonstrated to cause lesions on rubber clones examined in this study. There was variation in susceptibility of the rubber clones to the pathogen. This study provides useful information that could be exploited to better manage the disease.

Keywords: *Neofusicoccum ribis, rubber, leaf blight disease, Hevea brasiliensis*

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1. Introduction

Rubber tree (*Hevea brasiliensis*) is a tropical crop from the Amazon forest of South America, named as 'rubber' by Priestly in 1770 [1]. The rubber plant (*Hevea brasiliensis*) is a natural source of rubber and an important plantation tree species. This plant represents a potential species for reforestation and commercial exploitation programmes in tropical countries [2].

Hevea brasiliensis was introduced from Brazil to South East Asia in 1876. Of the 70,000 rubber seeds brought from the Amazon forest in Brazil to Kew Garden, England, Botanical Garden, Ceylon, and Singapore Botanical Garden by three British men, Farris, Cross and Henry Wickham, nine seeds were transported to Kuala Kangsar, Perak, successfully raised and distributed in Malaya (present Malaysia) [1]. The first rubber exploitations were set up in Southeast Asia from 1890 to 1930 [3]. South East Asia is the main producer of natural rubber in the world [1]. Currently, there are more than 30 clones of rubber trees planted in Malaysia [1].

In order to sustainably maintain quality rubber production, there is the need to develop better quality planting material through more efficient rubber tree breeding and propagation processes [4]. In this context, efficient disease management strategies are crucial in modern rubber growing. Rubber production potential is strongly threatened by leaf blight caused by *Neofusicoccum* sp. [5]. A species of *Neofusicoccum* was isolated in Malaysia from diseased rubber leaves showing *Fusicoccum* leaf blight (FLB), with large lesions and concentric brownish zones as well as dark pustules on the upper leaf [6].

Despite the importance of attacks by the FLB pathogen on commercially grown rubber, variations in susceptibility among clones as a method to improve the management of this disease have not been examined. The aim of this study was therefore to identify and evaluate the pathogenicity of various *Neofusicoccum* isolates on both detached leaves *in vitro* and leaves of seedlings of different rubber clones *in vivo* to determine whether clonal variation could be used to better manage the disease problem.

2. Materials and Methods

Neofusicoccum isolates were obtained in 2010 from diseased leaves of rubber clones showing typical symptoms of blight in three states: Selangor (Sungai Buloh), Perak (Sungai Biong), and Johor (Segamat and Kota Tinggi) of peninsular Malaysia [5]. Samples (Table 1) were collected from rubber trees representing seven clones in all three locations.

Depending on the availability of clones at each location, at least 5 trees per clone were randomly chosen, ten leaves were cut per tree, and all the samples were placed in paper bags and transported to the laboratory where they were processed within one day.

Table 1. Isolates of *Neofusicoccum ribis* used in pathogenicity tests.

Code	GenBank ITS	Host	Year of planting	State	Location
SB30	JX035740	RRIM 2024	2004	Selangor	Sungai Buloh
SK10	JX035744	PB 350	2004	Perak	Sungai Kapis
SJ20	JX035769	RRIM 2023	2005	Johor	Kota Tinggi

Isolation of the pathogen was made from lesion margins of diseased leaves onto Potato Dextrose Agar (PDA) (Difco, Maryland-USA) supplemented with 1 mg/mL of streptomycin and incubated for 7 days at 26°C under near UV-light. The emergent isolates were purified by three successive subcultures for morphological characterization.

Inoculation with three representative isolates (Table 1), one per locality (SB30, SJ20 and SK10) was realized to investigate their pathogenicity on the clonal lines from which they were originally isolated and on other common rubber clones (RRIM 2002 , RRIM 2023, RRIM 2025, RRIM 2024, RRIM 2007 , PB 260 and PB 350). Fungal mycelium was grown on PDA to produce conidia [5]. Conidial suspension was prepared 24h before each inoculation day at 25°C and the concentration was adjusted to 3 x 10^5 spores/mL using a haemocytometer.

A detached leaf method arranged in completely randomized design (CRD) was used for pathogenicity test *in vitro*. A single two week-old leaf was placed in a 15 cm-diameter Petri dish [7]. Four leaves per clone were inoculated by placing ten droplets of 10 µL of the spore suspension onto the abaxial side. The leaves were then placed under continuous fluorescent light (20°C) at 70-80% relative humidity. Control leaves were inoculated with distilled water.

On rubber seedlings grown in a greenhouse, ten 12-day old leaflets of each plant were inoculated by spraying 50 mL of the fungal conidial suspension early in the morning in the CRD. Thereafter, for each seedling, dampened cotton wool was wrapped around the central internodes of the stem and covered with a white, moist polythene bag to create favorable conditions for infection. The polythene bag was removed after 24 hours in the same greenhouse and the inoculated seedlings were maintained for two days at the same temperature [(24°C (night) / 28°C (day) and relative humidity: 70-80%)]. Seedlings sprayed with distilled water were used as control. For both detached leaf and seedlings methods, three replicates were conducted for each isolate used, and the effect of the fungal isolate on each rubber clone was analyzed concurrently.

The blight was assessed at 5, 10 and 15 days intervals for the detached leaves, and at 30 days for the seedlings. The disease was scored for detached leaves on a scale of 0-4 as described in Table 2. On seedlings, specifications on reaction were scored with the same scale, following the percentage of leaves infected [8].

Table 2. Disease scale description

Method used	Scale	Descriptions
Detached Leaves	0	No visible lesion
	1	Light lesion
	2	Dark lesion
	3	Large lesion with sporulation
	4	Very high sporulation
Seedlings	0	No observable change or lesion
	1= (1-5%)	1 leaf with discoloration
	2= (5-25%)	1 to 3 leaves with lesions
	3=(25-50%)	3 to 5 leaves with lesions
	4= >50%	More than 5 leaves with lesions

The disease severity index (DSI) for each leaf was calculated using the following formula [9]:

$$DSI = \left(\sum_{i=0}^{4} N_i \times i \right) \Big/ \sum_{i=0}^{4} N_i$$

where i is severity (0-4) and Ni is the number of leaves with the severity of i.

The Percentage Disease Intensity (PDI) was calculated as follows: (Number of lesions developed/number of total inoculated spots) x 100%. Percentage of disease incidence was transformed to arcsine and analyzed for sources of variance (ANOVA). Tukey's procedure, Honest significant different (HSD) at P = 0.05 was used for the comparison of mean values of lesion size between treatments. All analyses were done as described by [10] by using IBM SPSS software version 17.

Fungal genomic DNA was extracted from mycelia harvested by scraping the fungal colonies with a glass slide [11]. In order to characterize the 33 isolates, the oligonucleotide primers ITS1 and ITS4 [12,13] were used to amplify and sequence the internal transcribed spacer regions (ITS1 and ITS2) as well as the complete 5.8S gene. Another locus (BotF15) containing microsatellite repeats amplified with primers BOT15 and BOT16 [14] was also sequenced to obtain additional information for two isolates representative per location of the three regions, then were read with MEGA version 4.0 [14]. The BotF15 data set consisted of 18 isolates, 6 of which were from rubber and 12 from the *N. ribis/parvum* species complex, representing the most complete database of BotF15 sequences in this family [13,16]. Polymerase Chain Reaction (PCR) was used to amplify each gene region [16,17]. Phylogenetic analysis using PAUP [17] to determine relationships within individual and combined data sets was performed on the sequences of closely related species in the *N. ribis/parvum* species complex available from GenBank. A sequence of *Guignardia philoprina* was used as an outgroup. The support for branches of the most parsimonious trees was assessed [19] and other parameters considered were the tree length, consistency index, rescaled consistency index and retention index [20].

3. Results and Discussion

A total of 105 trees were sampled at three localities. From a total of 68 isolates obtained, 33 were identified to belong to the taxon Botryosphaeriaceae. The colony texture and colour was either white to grey or cottony faint white and produced only anamorph structures. Initially, the ellipsoidal conidia were hyaline and aseptate (9.0-25.5 µm (length) x 2.1-8.5 µm (width), L/W ratio of 3.4) which with age, changed to light brown with one or two septa.

Results of ITS and BotF15 sequences, approximately and respectively 550 bp and 350 bp in size, were obtained after making consensus strand and editing of sequences.

These sequences were deposited in the NCBI GenBank database.

Isolates from the rubber were grouped in a single clade with *N. ribis* (CMW 7772 and CMW 7773). The tree generated from the BotF15 data also clustered the isolates from the rubber within the *N. ribis* and *N. umdonicola* clades. Concordance among the ITS and BotF15 data sets was confirmed by the calculated incongruence length difference (I = 0), which suggested a lack of conflict between these gene genealogies. The data sets were combined, and a total of 867 bases were generated for the combined ITS and BotF15 data sets. After heuristic searches, one of the most parsimonious trees of 19 steps (CI = 1, RI = 1, and RC = 1) was obtained. The consensus tree obtained from the combined analysis of ITS and BotF15 sequences showed that the isolates collected from diseased rubber leaves formed a clade with *N. ribis* (89%) (Figure 1). Three isolates of *N. ribis* used for the pathogenicity test were deposited into the International Collection of Microorganisms from Plants (ICMP) in New Zealand as ICMP 20076, ICMP 20077 and ICMP 20078.

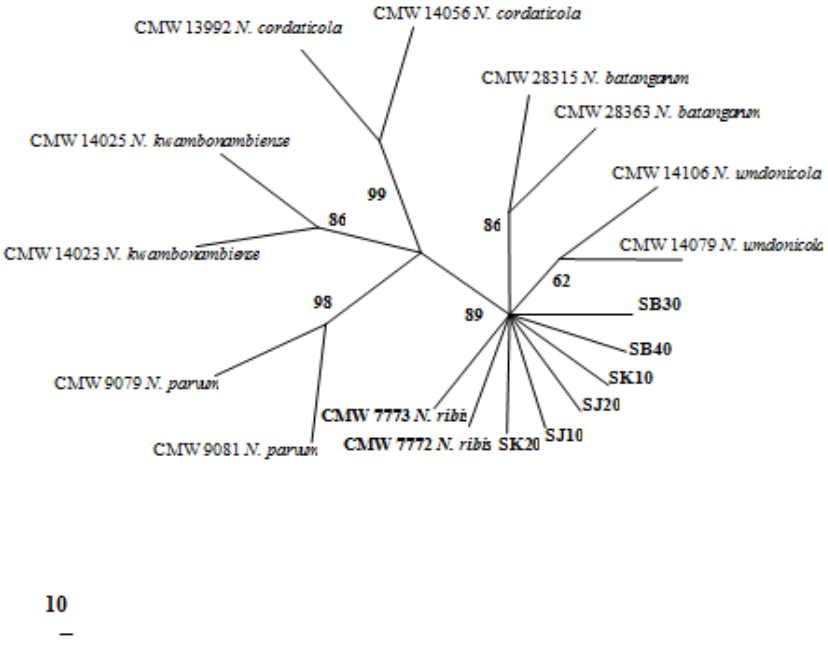

Figure 1. Phylogenetic tree based on combined sequences ITS and BotF15. Most parsimonious unrooted tree inferred from Maximum Parsimony analyses of the combined ITS and BotF15 sequence data of the representative taxa of the Botryosphaeriaceae [5]. The scale shows 10 changes in nucleotide and the Bootstrap support (%) from 1000 replications is given on the branches. Isolates marked in bold represent those from rubber

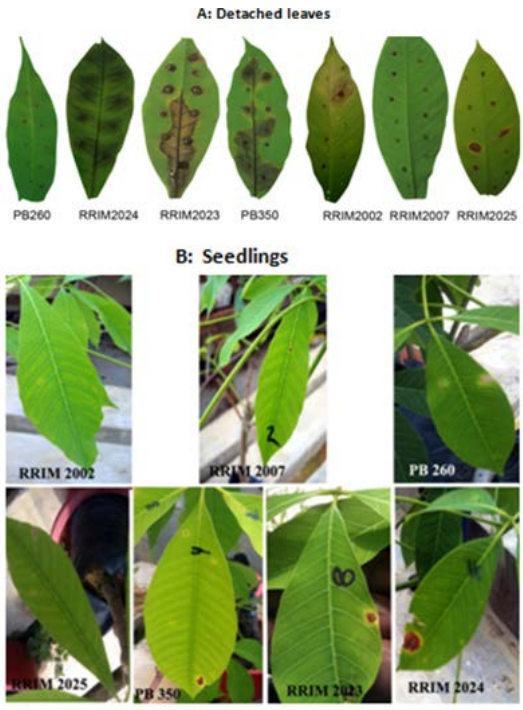

Figure 2. Levels of infection of *Neofusicoccum ribis* on detached rubber leaves 15 days post inoculation (A) and on seedling leaves 30 days post inoculation (B)

Small, light spots were visible on detached leaves 5 days post inoculation (dpi) for all rubber clones. Undulated margins with pycnidia on the leaf surface appeared at 10 dpi which turned completely brown at 15 dpi (Figure 2A). However, a highly significant effect on the DSI resulting from the interaction between the isolates and rubber clones after inoculation was observed ($P < 0.05$). At five dpi, isolate SB30 gave the smallest lesions compared to isolates SJ20 and SK10. Moreover, all isolates tested on clone RRIM 2002 and RRIM 2007 showed less infection for all intervals evaluated (Table 3). In contrast, clones RRIM 2024 and PB 350 were more severely affected by all isolates tested at 15 dpi ($P < 0.05$).

Rubber seedlings responded differently to different fungal isolates compared to control. However, the percentage of disease incidence on seedling leaves was lower than that on detached leaves 30 dpi (Figure 2B), and isolate SK10 was the most pathogenic. Clones RRIM 2023, RRIM 2024 and PB 350 showed higher infection (>50%) while lower infection (<25%) was recorded for the RRIM 2002 and RRIM 2007 clones (Figure 3).

After inoculation, lesions were fully developed after one month, extending upwards and downwards. Wavy concentric brownish zones appeared and embedded black pycnidia were produced within the lesions.

Table 3. Pathogenicity (% Disease Intensity (PDI) of *Neofusicoccum ribis* isolates on different rubber clones using detached leaf assay assessed at 15 days post inoculation

Isolate	Percentage Disease Intensity (PDI) (%) and Mean score						
	PB 260	RRIM 2024	RRIM 2023	PB 350	RRIM 2007	RRIM 2002	RRIM 2025
SB30	18.33(0.73)^{ab}	39.17(1.57)^{b}	29.44(1.18)^{ab}	38.89 (1.56)^{b}	6.67 (0.27)^{a}	12.50 (0.5)^{ab}	30.28(1.21)^{ab}
SJ20	29.72(1.19)^{ab}	56.11(2.24)^{b}	14.72 (0.59)^{a}	45.28(1.81)^{ab}	28.0(1.12)^{ab}	13.89 (0.56)^{a}	31.11(1.24)^{ab}
SK10	25.56(1.02)^{ab}	51.11(2.02)^{b}	49.72(1.99)^{ab}	52.50 (2.10)^{b}	18.89 (0.76)^{a}	16.94 (0.68)^{a}	26.94(1.08)^{ab}

For each isolate, mean of PDI (Percentage Disease Intensity) in same row with the same letter are not significantly different at P=0.05 according to Tukey HSD test. The numbers in parentheses represent the standard deviation of the average infection score.

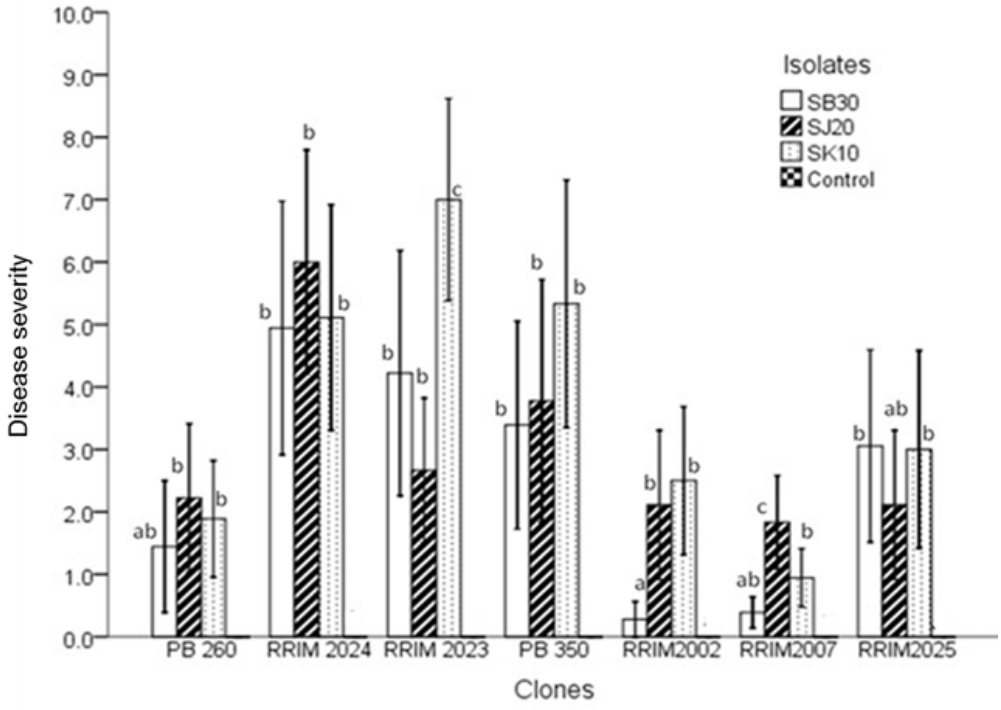

Figure 3. Disease severity of *Neofusiccocum ribis* isolates on different clones of rubber seedlings 30 days post inoculation. Bars represent the standard deviation of the mean lesion lengths which were significantly different (P <0.05) according to Tukey HSD test

4. Conclusion

The results of inoculation trials conducted in this study showed that all *N. ribis* isolates tested were pathogenic to young leaves of rubber and seedlings. However, the virulence of these isolates varied depending on the rubber clone. Based on the average Percentage Disease Intensity (PDI), rubber clones tested were classified into three categories based on their ability to sustain infection following artificial inoculation: Group 1: susceptible clones (PDI > 10%) including RRIM 2023, RRIM 2024 and PB 350; Group 2: moderately resistant clones (PDI > 5%) including PB 260, RRIM 2025, and Group 3: resistant clones (PDI < 5%) including RRIM 2002 and RRIM 2007. The conidia of *N. ribis* were successfully re-isolated from infected leaves of seedlings that showed symptoms of leaf blight.

Although *N. ribis* is considered to be a tropical plant endophyte [13], we demonstrated the pathogenicity of *N. ribis* on rubber seedlings. Future studies need to be conducted to investigate the ecology of the fungus in environments where it prevails focusing on factors that influence pathogenicity in order to develop more effective quarantine strategies.

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References

[1] Noordin W. D., Shafar J. M. and Che F. I., 2012. Assessment of selected *Hevea brasiliensis* (RRIM 2000 Series) seeds for rootstocks production. *African Journal of Agricultural Research* 7 (21): 3209-3216.

[2] Hernández R. C. A., Kafuri L. A., Isaza R. A. and Arias M. L., 2006. Analysis of genetic variation in clones of rubber (*Hevea brasiliensis*) from Asian, South and Central American origin using RAPDs markers. *Rev. Colomb. Biotecnol.* 8 (2): 29-34.

[3] Dibi K., Boko C., Obouayeba S., Gnagne M., Dea G. B., Carron M. P. and Anno A. P., 2010. Field growth and rubber yield of in vitro micropropagated plants of clones PR 107, IRCA 18 and RRIM 600 of *Hevea brasiliensis* (Muëll.-Arg.). Agric. *Biol. J. N. Am.* 1 (6):1291-1298.

[4] Montoro P., Carron M. P., Lardet L., Demange A. C., Leclercq J., 2010. Biotechnologies in rubber tree (*Hevea brasiliensis*). *AsPac J. Mol. Biol. Biotechnol.* 18 (1): 81-83.

[5] Nyaka Ngobisa A. I. C., Zainal Abidin M. A., Wong M. Y. and Wan Noordin M. W. D., 2013. *Neofusicoccum ribis* Associated

with Leaf Blight on Rubber (*Hevea brasiliensis*) in Peninsular Malaysia. *Plant Pathology Journal* 29 (1): 10-16.

[6] Radziah N. Z. and Chee K. H., 1989. A new foliar disease of rubber. *Plant Pathology* 38: 293-296.

[7] Ismail H. and Jeyanayagi I., 1999. Occurrence and identification of physiological races of *Corynespora cassiicola* of Hevea. *In*: Proceedings of the IRRDB Symposium 1999. (Eds QB Chen, JN Zhou). Hainnan Publishing House: Haikou. pp. 263-272.

[8] Manju M. J., Vinod, K. K., ldicula S. P., Kuruvilla J. C., Nazeer M. A and Benagi V. I., 2010. Susceptibility of *Hevea brasiliensis* clones to Corynespora Leaf Fall disease. *Mycology Plant Pathology* 40 (40): 603-609.

[9] Ma Z. and Michialides T. J., 2002. Characterization of *Botryosphaeria dothidea* Isolates Collected from Pistachio and Other Plant Hosts in California. *Phytopathology* 92 (5): 519-526.

[10] Nei C., Hull J., Jenkins D., Steinberger D. and Bent N., 1975. 'SPSS: statistical package for the social sciences 11.0' (McGraw-Hill: Chicago, IL).

[11] Moller E. M., Bahnweg G., Sandermann H. and Geiger H. H., 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Research* 20: 6115-6116.

[12] White T. J., Bruns T., Lee S. and Talor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis M. A., Gelfand D. H., Snisky J. J. and White T. J. (Eds), PCR protocols, a guide to methods and applications. New York, USA: *Academic press*, pp 315-322.

[13] Begoude B. A. D., Slippers B., Wingfield M. J. and Roux J., 2010. Botryosphaeriaceae associated with *Terminalia catappa* in Cameroon, South Africa and Madagascar. *Mycological Progress* 9 (1): 101-123.

[14] Slippers B., Fourie G, Crous P. W., Coutinho T. A., Wingfield B. D., Carnegie A. J., Wingfield M. J., 2004b. Speciation and distribution of *Botryosphaeria* spp. on native and introduced Eucalyptus trees in Australia and South Africa. *Stud. Mycol*. 50: 343-358.

[15] Tamura K., Dudley J., Nei M. and Kumar S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*. 24: 1596-1599.

[16] Pavlic D., Slippers B., Coutinho T. A., Wingfield M. J., 2009. Molecular and phenotypic characterization of three phylogenetic species discovered within the *Neofusicoccum parvum*/*N. ribis* complex. *Mycologia* 101 (5): 636-647. doi: 10.3852/08-193

[17] Pavlic D., Slippers B., Coutinho T. A., Wingfield M. J., 2009. Multiple gene genealogies and phenotypic data reveal cryptic species of the Botryosphaeriaceae: A case study on the *Neofusicoccum parvum*/*N. ribis* complex. *Mol. Phylogenet. Evol*. 51: 259-268.

[18] Swofford D. L., 2002. PAUP. Phylogenetic Analysis Using Parsimony (and other methods). Version 4.0b10. Sunderland, MA, USA: Sinauer Associates.

[19] Felsenstein J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.

[20] Hillis D. M. and Huelsenbeck J. P., 1992. Signal, noise and reliability in molecular phylogenetic analyses. *Journal of Heredity* 83: 189-195.