

Laboratory tests to assay responses of rubber (*Hevea brasiliensis*) genotypes to *Phytophthora meadii*

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Abstract

Variability in response to Phytophthora infections by different rubber (Hevea brasiliensis) genotypes is attributed to biochemical reactions occur in petioles upon infections. Latex is the main product of the rubber tree which is present in all tissues possibly contributing to the biochemical responses. Latex serum from tolerant genotypes significantly stimulated germination of P. meadii zoospores, while that from susceptible genotypes did not stimulate. Extracts from P. meadii-infected petioles of tolerant genotypes significantly reduced P. meadii zoospore germination, while Mycelial growth on liquid medium was significantly inhibited by the healthy petiole extracts of tolerant genotypes. The study explored the possibility of using these criteria for laboratory assay of new rubber genotypes to P. meadii.

Keywords: *Hevea brasiliensis*, leaf disease, resistance

Introduction

Hevea brasiliensis (A. Juss.) Muell. Arg. (rubber tree) is infected by several pathogens and the major pathogens include *Phytophthora meadii* McRae and other species, *Corynespora cassiicola* (Berk & Curt.), *Colletotrichum gloeosporioides* (Penz.) Sacc., *C. acutatum* Simmonds ex Simmonds, *Oidium heveae* Steinm. Immature rubber plants in nurseries are

susceptible to most of the above pathogens, whereas mature trees in plantations resist some of the pathogens such as *C. cassiicola*. The tolerance of one genotype to a certain disease is a unique genetic trait. Therefore, defence-related biochemical factors are important to be evaluated towards building up a relation to the resistant level of genotypes to *P. meadii*.

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Although mechanisms of plant resistance are still not fully understood, it is known to be under genetic control (Kombrink & Somssich, 1997). Amongst many resistance related activities, synthesis of PR proteins is an important plant defence mechanism (Kombrink & Somssich, 1995), while Phenylalanine Ammonia-Lyase (PAL) and oxidase activities are indicators of host resistance (Narasimhan *et al.*, 2000). Cinnamyl-alcohol dehydrogenase and isoperoxidases are also known as important in plant resistance since, they were found as increased amounts in infected rubber roots (Nicole *et al.*, 1985) while, Scopoletin was observed in leaves infected by *C. gloeosporioides* (Giesemann *et al.*, 1986). PR-proteins (Narasimhan *et al.*, 2000) and several phenolics in petioles (Jayasuriya *et al.*, 2003) have also been related to resistance of rubber to *P. meadii*. However, formation of lignin has been noticed as an important tolerant reaction of the resistant genotype RRIC100 upon *P. meadii* infection. Vanillin was also found as prominent phenolic substance in petioles of RRIC100 (Jayasuriya *et al.*, 2003).

The rubber genotype RRIC 121 which is susceptible to *Phytophthora* leaf fall disease but resist bark infection caused by the same pathogen (Jayasinghe & Wettasinghe, 1997). This suggests the involvement of many factors in the resistance response of rubber against *Phytophthora*. This paper

explores the possibility of using some biochemical responses involved in the resistance of rubber to selected *P. meadii* strains, for the development of a laboratory assay to determine the level of resistance of rubber genotypes to *P. meadii*.

Materials and methods

Pathogen isolates

A virulent *P. meadii* isolate MAD86 (IMI385259), and an avirulent DF600 isolate (IMI 385260) obtained from infected petioles of PB86 and RRIC600 genotypes respectively (Jayasuriya *et al.*, 1999) were used throughout the investigation.

Plant material

Four-year-old trees of the following genotypes grown in the premises of the Rubber Research Institute were used; RRIC100 (tolerant) (Jayasinghe, 1992), RRIC121 (susceptible) (Jayasinghe, 1995), BPM24 (tolerant) (Jayasinghe, 1995), PB86 (susceptible) (Jayasinghe, 1996), RRIC600 (susceptible) (Jacob *et al.*, 1989) to the leaf fall disease caused by *Phytophthora*.

Inoculation of petioles with P. meadii

Mature petioles were obtained from five-year-old trees of each genotype and the cut ends were sealed off with molten paraffin. Thereafter, the petioles were surface sterilised, kept in plastic trays and inoculated with a

zoospore suspension (10^4 zoospores ml^{-1}) obtained from the isolates MAD86 or DF600 and incubated at $27\pm 2^\circ\text{C}$ for 72 h as described previously (Jayasuriya *et al.*, 1999).

Determination of the effect of latex serum from different rubber genotypes on germination of P. meadii zoospores

The effect of latex serum against *P. meadii* was tested to determine if any relationship exist between latex and petiole infections. Latex was obtained during early hours of the day. Latex collected (10 ml) from at least 5 field trees (4-year-old) was centrifuged at 15,000 g for 30 min (Beerhues *et al.*, 1994) in 1.5 ml microfuge tubes. Serum was obtained using a syringe and filtered through a Millipore filter (Nalgene[®], 0.2 μm) and used immediately or stored at -20°C until use. This serum is referred in the text as sterilised serum.

A 2.9 ml of zoospore suspension (10^4 zoospores ml^{-1}) from MAD86 was mixed with 0.1 ml of sterilised serum in McCartney bottles at $27\pm 2^\circ\text{C}$. Zoospore germination was suspended after 1.5 h by adding 1 drop of Cotton Blue in lactophenol to each bottle. A zoospore suspension similarly mixed with sterilised distilled water served as the control. Drops of the suspensions were thereafter mounted on glass slides and the germination of randomly selected 25 zoospores was microscopically ($\times 100$) assessed. Zoospores having germ tubes longer than their breadths were considered as

germinated. Four replicate slides were prepared for each suspension and the experiment was repeated twice and results were pooled.

Determination of the effect of extracts from healthy or infected rubber petioles on P. meadii

This investigation was carried out to extract soluble antifungal phenolic compounds from healthy and infected rubber petioles. Excised petioles (obtained from the top whorls of at least 10 trees of each genotype) were immediately washed with sterile distilled water and the excess water was drained off. Small pieces cut from the middle portions (40g) were homogenised with 50% (v/v) boiling ethanol (Harborne, 1989). The homogenate was kept overnight at 4°C and thereafter centrifuged at 3000 g for 10 min. The supernatant was filtered and the residue was re-extracted. The pooled filtrate was dried by rotary evaporation and the residue re-dissolved in absolute ethanol was sterilized by Millipore (NALGENE[®], PES 0.2 μm) filtration. Extracts were used immediately or stored at -20°C until use.

Zoospores from MAD86 were obtained from cultures as described previously (Jayasuriya *et al.*, 1999). Test extract (1 μl) was added to 30 μl of sterilised distilled water containing 10^2 zoospores ml^{-1} on a sterilised glass slide. The slide was incubated for 1 h in a closed Petri plate at 26°C and a drop of cotton blue in lactophenol was added.

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Fifty randomly selected zoospores were observed for germination under $\times 100$ magnifications. In the control, 1 μ l of absolute methanol was used instead of the test extract. Assessments were repeated 5 times using 500 zoospores in each instance.

Determination of the effect of petiole extracts on the growth of P. meadii on PDA

Growth was examined in pea broth (De Cock *et al.*, 1992) using the method described by Yoshikawa (1978) after modification. One ml of the test extract was added to 30 ml of pea broth in a 125-ml conical flask. The broth was inoculated with one mycelial disc (5 mm) obtained from the edges of an actively growing *P. meadii* cultures. Thereafter, the flasks were incubated at $27\pm 2^\circ\text{C}$ for 8 days after which the mycelia were harvested by vacuum

filtration. Mycelia were oven-dried at 80°C and weighed and the inhibition of the growth was defined as the loss of dry matter against the controls. The control was grown in 31 ml pea broth. Results were expressed as mg of mycelium per 31 ml of medium. Experiments were repeated at least 3 times employing at least 8 replicates each time.

Results

Effect of latex serum on the germination of P. meadii zoospores

Latex serum from resistant genotypes significantly ($P<0.05$) stimulated zoospore germination (by 142-177 %), while serum from susceptible genotypes either did not increase (in RRIC121 and PB86) or significantly ($P<0.05$) reduced (in RRIM600) zoospore germination compared to the control (Table 1).

Table 1. *Effect of H. brasiliensis latex serum on the germination of P. meadii zoospores*

Genotype (response to <i>Phytophthora</i>)	% Germination in serum	% Germination compared to control
RRIC 100 (t)	25.0 \pm 1.91	277.7
BPM 24 (t)	21.8 \pm 1	242.2
RRIC 121 (s)	9.0 \pm 1.91	100.0
PB 86 (s)	9.0 \pm 1	100.0
RRIM 600 (s)	5.0 \pm 1	55.5
Control	9.0 \pm 1.91	100
	LSD = 0.0817	

Values are means of 100 replicates \pm SEM. t = tolerant, s = susceptible.

Effect of the extract from healthy petioles on germination of *P. meadii* zoospores

Test extracts from healthy, resistant genotypes significantly ($P<0.05$) inhibited germination of *P. meadii* zoospores than extracts from the susceptible genotypes. The OD₃₈₀ values of the extracts from healthy petioles of RRIC100 and BPM24 were equal and also higher than the OD₃₈₀ values of similar extract of susceptible genotypes (Table 2).

Effect of extracts from *P. meadii*-infected petioles on germination of *P. meadii* zoospores

Extracts from infected petioles of resistant genotypes have significantly

($P<0.05$) inhibited germination of zoospores (Table 2).

Effect of petiole extracts on growth of *P. meadii*

The effect of petiole extracts on the growth of *P. meadii* was not always consistent. Extracts from healthy petioles of susceptible genotypes significantly ($p<0.05$) increased the mycelial growth in the liquid medium. However, the extracts from infected petioles of the same group did not increase the growth. In majority of cases, the effect on the growth of was significantly ($p<0.05$) lower when the medium was amended with the extract from infected petioles (Table 3).

Table 2. Effect of extracts from healthy or *P. meadii*-infected petioles on germination of *P. meadii* zoospores

Genotype (response to <i>Phytophthora</i>)	Extract from healthy petioles		Extract from infected petioles	
	OD ₃₈₀ [#]	% germination (% inhibition)	OD ₃₈₀ [#]	% germination (% inhibition)
RRIC100 (t)	2.89±0.002 (1.5)	67.2 ^d (24.5)	0.05±0.008 (0.3)	61.5 ^d (30.9)
BPM24 (t)	2.86±0.003 (1.4)	72.5 ^d (18.5)	1.99±0.006 (1.4)	64.3 ^d (27.7)
RRIC121 (s)	1.84±0.001 (1.3)	83.8 ^b (5.8)	1.33±0.001 (1.0)	87.1 ^a (2.13)
PB86 (s)	1.62±0.001 (1.2)	77.5 ^c (12.9)	1.23±0.002 (0.9)	68.9 ^c (22.6)
RRIM600 (s)	1.53±0.002 (1.2)	84.2 ^b (5.4)	1.16±0.007 (0.8)	72.4 ^b (18.6)
Control		89.0 ^a		89.0 ^a

#Values are means of 4 replicates ± SEM. #Values in parentheses indicate approximate concentrations (mg ml⁻¹) of phenolic compounds in each extracts. Values sharing common letters in columns do not differ significantly according to the Duncan's Multiple Range Test. t = tolerant, s = susceptible.

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Table 3. Effect of petiole extracts of rubber on the mycelial growth of *P. meadii* (MAD86) in Pea broth

Genotype (response to <i>Phytophthora</i>)	Extract from healthy petioles		Extract from infected petioles		LSD
	Biomass g	% Inhibition	Biomass g	% Inhibition	
RRIC100 (t)	0.21 ± 0.003 ^a	8.7	0.22 ± 0.005 ^a	4.3	0.015
BPM24 (t)	0.19 ± 0.002 ^a	17.4	0.21 ± 0.006 ^a	8.7	0.017
RRIC121 (s)	0.28 ± 0.005 ^{a*}	0	0.23 ± 0.006 ^b	0	0.019
PB86 (s)	0.29 ± 0.01 ^{a*}	0	0.21 ± 0.01 ^b	8.7	0.035
RRIM600 (s)	0.28 ± 0.007 ^{a*}	0	0.24 ± 0.01 ^b	0	0.035
Control	0.23 ± 0.002				

Values are means of 24 replicates ± SEM. ‘*’ mark indicates increased growth than control. Results marked with ‘**’ in column 2 from left are significantly ($p < 0.05$) different according to t-test. Means with same letter in lines (results pertaining to either healthy or infected) are not significantly different according to Duncan’s Multiple Range Test. t = tolerant, s = susceptible.

Discussion

Due to the non-availability of more clones established either as resistant or susceptible to diseases caused by *Phytophthora*, only five rubber genotypes were used in this investigation. The susceptible genotypes used in the study had similar characteristics, which significantly varied from the characters of the tolerant genotypes. Latex serum from tolerant genotypes had pronounced effect on zoospore germination which probably can be attributed to sugars or proteins in the serum as sugars and proteins are known to promote zoospore germination of *Pythium* spp (Donaldson & Deacon, 1993). However, although a variation among the total protein content in serum of rubber clones was not observed, a difference in basic protein pattern had been reported

(Premathilake *et al.*, 1985; Premathilake & Yapa, 1985). Therefore, the serum test would be reliable to assay the difference between tolerance and susceptible genotypic effect against *P. meadii*.

The toxic reaction of tolerant genotypes against *P. meadii* is probably due to different phenolic substances contained in petioles. The tolerant types are reported to contain more fungitoxic substances such as vanillin (3-methoxy-4-hydroxybenzaldehyde) (Jayasuriya *et al.*, 2003) and other coumarins (Gieseman *et al.*, 1986). The lower effect of extracts from *P. meadii* infected petioles may be due to polymerization of toxic phenolic monomers upon infection and the formation of insoluble compounds such as lignin. This is apparent from lower OD₃₈₀ values observed from *P. meadii*

infected petiole extracts. Unpublished results available in the Department of Plant Pathology & Microbiology of the Rubber Research Institute of Sri Lanka indicated clear and thick lignin deposits in infected areas of RRIC100 petioles.

These measurements or analyses of *P. meadii* zoospore germination percentages were earmarked upon confrontation with latex serum or petiole extracts from rubber genotypes known as either tolerant or susceptible to *P. meadii*. Such particular measurements could also be used as criteria to assay the tolerance response of new rubber genotypes to *P. meadii*. However, it may be worthwhile to explore the possibility of using this technique to assay the resistance response of *Hevea brasiliensis* genotypes against other leaf diseases too.

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