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Variability in defence responses of rubber genotypes against *Phytophthora meadii* infections

K E Jayasuriya* and R L C Wijesundera**

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Abstract

The possibility of using the biochemical responses of the rubber plant as criteria for assessing tolerant levels against P. meadii *was investigated. Synthesis of PR-proteins and PAL enzyme were examined for any relationship to tolerance against* P. meadii *infection. PR-proteins were produced 24 h after inoculation in tolerant petioles whereas in susceptible petioles it was only 48 h after inoculation. In infected attached petioles of tolerant plants, PR protein levels were also higher than that of susceptible plants. The activity of PAL was higher in tolerant infected petioles than that of other genotypes. These criteria could be used to differentiate rubber genotypes tolerant to* P. meadii.

Keywords: *Hevea brasiliensis*, *Phytophthora* leaf disease, PR-proteins, tolerance

Introduction

Hevea brasiliensis (A. Juss.) Muell. Arg. (rubber tree) is infected by pathogens causing die-back, leaf fall with subsequent yield losses. Among them, *Phytophthora meadii* McRae, *P. palmivora* (Butl.) Butl., *Corynespora cassiicola* (Berk & Curt.), *Colletotrichum gloeosporioides* (Penz.) Sacc., *C. acutatum* Simmonds ex Simmonds, *Oidium heveae* Steinm are important. Mature trees tolerate some pathogens, though susceptible to same in nurseries. Further, disease occurrence in one genotype due to certain pathogens in one agro-climatic locality differs from that of another locality, indicating that certain climatic conditions are not conducive for certain diseases. Therefore, before a new rubber genotype is released to growers, researchers have to be confident of its tolerance levels against the majority of common diseases. To screen new rubber genotypes against diseases, it is essential to conduct series of experiments in different agro-climatic localities which is time consuming and expensive. The level of resistance of a genotype for one disease is a unique genetic trait. Therefore, defence-related

* Rubber Research Institute of Sri Lanka, Dartonfield, Agalawatta, Sri Lanka
** Faculty of Science, University of Colombo, Colombo 3, Sri Lanka

biochemical factors should be evaluated for any relationship to resistant levels of genotypes to *P. meadii* and other pathogens.

Although the biochemistry of plant resistance is still not completely understood, both host specificity and resistance are known to be under genetic control (Kombrink *et al.*, 1997). Synthesis of PR proteins is known as an important biochemical plant defence response (Dixon & Harrison, 1990; Kombrink & Somssich, 1995), while Phenylalanine Ammonia-Lyase (PAL) including polyphenoloxidase activities are often considered as indicators of resistance of the host plant (Narasimhan *et al.*, 2000). Specifically, peroxidases are involved in the resistance response by polymerising phenolics to lignin (Simons & Rose, 1971; Harborne, 1989).

In *H. brasiliensis*, increase of cinnamyl-alcohol dehydrogenase, isoperoxidases have been observed upon infection by root pathogens (Nicole *et al.*, 1985). Scopoletin was observed in rubber leaves responding to *C. gloeosporioides* (Tan & Low, 1975; Giesemann *et al.*, 1986). PR-proteins have been identified as an important factor in determining resistance in rubber (Narasimhan *et al.*, 2000) while a variation of terpenes, triterpinoides, flavonoides and phenolic acids in petioles were shown to be in rubber genotypes that are either resistant or susceptible to *P. meadii* (Jayasuriya *et al.*, 2003). However, formation of lignin from phenolic aldehydes is suspected as the resistant reaction of the resistant genotype upon infection. Vanillin was prominent in petioles of the resistant genotype RRIC 100, which also affected the germinating zoospores (Jayasuriya *et al.*, 2003). The genotype RRIC 121, which is susceptible to leaf disease is resistant to bark rot (Jayasinghe & Wettasinghe, 1997) caused by *Phytophthora*. This suggests an involvement of multiple factors in resistance expression in different rubber genotypes. Studies carried out so far have considered only few factors involved in compatible interactions among few genotypes. This paper reports factors involved in resistance of *H. brasiliensis* to *P. meadii* strains, which will be useful in designing an assay for resistance to *P. meadii* or other pathogens causing leaf diseases.

Materials and Methods

Pathogen isolates

A virulent *P. meadii* isolate MAD86 (IMI 385259), and an avirulent DF600 isolate (IMI 385260) obtained from infected petioles of PB 86 and RRIM600 genotypes respectively (Jayasuriya *et al.*, 1999) were used throughout this investigation for inoculation of petioles.

Plant material

Four-year-old trees of the following genotypes grown in the premises of the Rubber Research Institute were used: RRIC100, tolerant to leaf disease (Jayasinghe, 1992), RRIC 121, moderately susceptible to

leaf disease (Jayasinghe, 1995), BPM24, tolerant to leaf disease (Jayasinghe, 1995), PB86, susceptible to leaf disease (Jayasinghe, 1996), RRIM600, susceptible to leaf disease (Jacob *et al.*, 1989) caused by *Phytophthora*.

Inoculation of detached petioles with* P. meadii *zoospores

Matured petioles were excised from trees and the cut end was sealed with molten paraffin. Thereafter, they were surface sterilised using 70% ethanol using sterilized cotton wool moistened with 70% ethanol and then rinsing with sterilized distilled water. They were kept in plastic trays and thereafter inoculated with aliquots (10 μl) of a zoospore suspension (10^4 zoospores ml^{-1}) obtained from the isolates MAD86 and DF600 and incubated at 27°±2°C for 72 h as described previously (Jayasuriya *et al.*, 1999). Four infected petioles from each genotype were collected at a time 24, 48, 72 or 96 h after inoculation for the extraction of PR-proteins.

Inoculation of attached petioles on field plants with* P. meadii *zoospores

For a comparison with the above experiment with detached petioles, live attached petioles were also used in a separate experiment. From each genotype randomly selected twenty mature petioles were surface sterilised using sterilized cotton wool moistened with 70% ethanol and thereafter rinsing with sterilized distilled water. Petioles were thereafter inoculated with absorbent cotton wool swabs moistened with 10 μl of the same zoospore suspension. Petioles similarly treated with sterilised distilled water served as the control. Inoculated petioles were tagged for easy identification.

Extraction of PR-proteins

Four infected petioles from each genotype were collected 24, 48, 72 or 96 h after inoculation for the extraction of PR-proteins. To avoid extracting proteins from *P. meadii*, tissues (3 g) were obtained from lesion margins and powdered in a pre-chilled mortar using liquid Nitrogen and extracted with 5 ml of extraction buffer (84 mM citric acid, 32 mM Na_2HPO_4, 14 mM β-mercapthoethanol and 6 mM ascorbic acid; pH 2.8) in McCartney bottles, while shaking at 200 rpm. After 1 h, the extracts were centrifuged at 15,000 *g* for 20 min (Tuzan *et al.*, 1989) and the supernatants were obtained. The extracts similarly obtained from petioles treated with sterilised distilled water served as control.

Detection of proteins by Spectrophotometer

Proteins in the extracts were determined according to Bradford (1976) using bovine serum albumin (BSA-Sigma) solution (5 mg in 25 ml of the extraction buffer) as the marker's stock solution. A concentration gradient of 0-200 μg of BSA was prepared using volumes of the stock solution and the

extraction buffer. Aliquots (100 μl) of BSA solutions were added to 5 ml Coomassie brilliant blue G-250 (Sigma) dye solution (100 mg dye in 50 ml of 95 % (v/v) ethanol to which 100 ml of H_3PO_4 was added and the whole mixture was diluted to 1000 ml with water and filtered). After 10 min, the absorbencies of BSA solutions were measured using a spectrophotometer (Camspec M330 UV/visible, 100-900 nm) at 595 nm. A standard curve was plotted with the absorbance against the concentrations of BSA. Proteins in the extracts obtained from infected petioles and reference (control) were detected by adding 25 μl of the extracted solution to 1.25 ml of the dye solution. Absorbencies were similarly measured, and the protein concentrations were determined using the calibration curve. This procedure was repeated three times for each extract. Protein levels of each time point in *P. meadii* infected petiole segments were assumed to be either similar (if PR-proteins are not formed due to infection) or higher (if PR-proteins are formed due to infection) and the differences of protein levels between infected tissues and references (controls) of the same clone at each time point were considered as PR-proteins, which can be considered as formed as a result of infection by *P. meadii*. PR-protein concentrations were calculated as μg proteins g^{-1} of fresh weight petioles.

Extraction of PAL enzyme

For extraction of PAL from infected petioles after different time points (24, 48 and 72 h) acetone-washed petiole powder was prepared from similarly infected or healthy petioles as previously described (Ferraris *et al.*, 1996). PAL was extracted according to a modified method (Jebakumar *et al.*, 2001) from 0.4 g of the petiole powder added with 15 ml of 0.1 M $Na_2B_4O_7$ buffer (pH 8.8) containing 20 mM β-mercapthoethanol. After shaking for 15 min at 300 rpm on an orbital shaker, the extract was purified by salting out with $(NH_4)_2SO_4$ to a final saturation of 46 % supernatant, while shaking for another 15 min. Then the solution was centrifuged at 15,000 *g* for 10 min and the precipitated proteins were dissolved in 4.5 ml of 0.1 M CH_3COONH_4 (pH 7.7).

Detection of PAL activity

PAL activity was measured according to a modified method (Ferraris, *et al.*, 1996). Two ml of the protein solution was added to 0.6 ml solution of 1 M *L*-phenylalanine (substrate) in a small McCartney bottle and the whole mixture was adjusted to 6 ml with 0.1 M CH_3COONH_4 (pH 7.7) and incubated in an air-blowing oven at 40°C for 1 h with a loosened cap. The conversion rate of *L*-phenylalanine into cinnamic acid was measured by spectrophotometer at 290 nm and the proportional amount of cinnamic acid produced was considered as a result of the PAL activity. The absorbance was extrapolated into the concentration of cinnamic acid in the solution using the

standard curve prepared as follows; Cinnamic acid (0.001 g) was dissolved in 100 ml of 0.1 M CH_3COONH_4 (pH 7.7). A concentration gradient was prepared using 1-10 ml of the above solution in 9-0 ml of sterilised distilled water respectively, resulting in 10-100 $\mu g\ ml^{-1}$ of cinnamic acid. Absorbance of solutions was measured at 290 nm using the same spectrophotometer and the standard curve of the concentration of cinnamic acid against absorbance (OD_{290}) was obtained. The activity of PAL was represented as mMol $h^{-1}\ g^{-1}$ fresh weight of petioles (0.35 units of acetone-washed petiole powder often equivalent to 1 unit of fresh petiole). The experiment was repeated four times using fresh samples. The average PAL activity and the variation in four samples were calculated statistically according to the procedure of general linear module in the SAS system. Initial PAL activities of each genotype were used as a covariant in the analysis and the means were separated by least square means (LSM).

Results

PR- proteins in P. meadii-*infected petioles*

PR-proteins were detected as the additional protein fraction produced in petioles upon administration with two *P. meadii* strains. In the majority of cases the amplitude of such proteins were higher in both attached and detached petioles which were infected with the aggressive MAD86 strain than in petioles which were infected with the avirulent DF600 isolate. In petioles of RRIC100, *in vitro* (Fig 1a) and *in planta* (Fig 1e) PR-protein amplitude increased up to 72 h and thereafter declined rapidly. A similar tendency was also seen in petioles of PB86 (Fig 1c), but the decline has been evident from 48 h onward *in planta* (Fig. 1g). The tendency of the increase of PR-proteins *in vitro* in detached petioles of RRIC121 (Fig. 1b) and RRIM600 (Fig. 1d) were similar with an increase from 24 h time point, but this increase has been evolved from zero or near zero values in both cases. However, the patterns of *in planta* (Fig. 1f, h) amplitudes of same petioles were not consistence, but upon infection with MAD86, higher PR-protein amplitudes were produced. In general, when comparing the PR-proteins produced in petioles upon infection by MAD86 after 24 h time point, the tolerant RRIC100 petioles had over 1.5 $\mu g\ ml^{-1}$ in detached (*in vitro*) petioles (Fig. 1a), and had over 2 $\mu g\ ml^{-1}$ in attached (*in planta*) petioles (Fig. 1e), although much lower values were detected in tolerant petioles infected with avirulent DF600 after 24 h time point. Petioles of susceptible clones after 24 h had much lower PR-protein values of 1 $\mu g\ ml^{-1}$ or lower (*in vitro*) or below 2 $\mu g\ ml^{-1}$ (*in planta*) (Fig. 1).

Phenylalanine Ammonia-Lyase (PAL) enzyme activity in petioles

In all cases, the PAL activity was significantly ($P<0.001$) and continuously higher in detached petioles

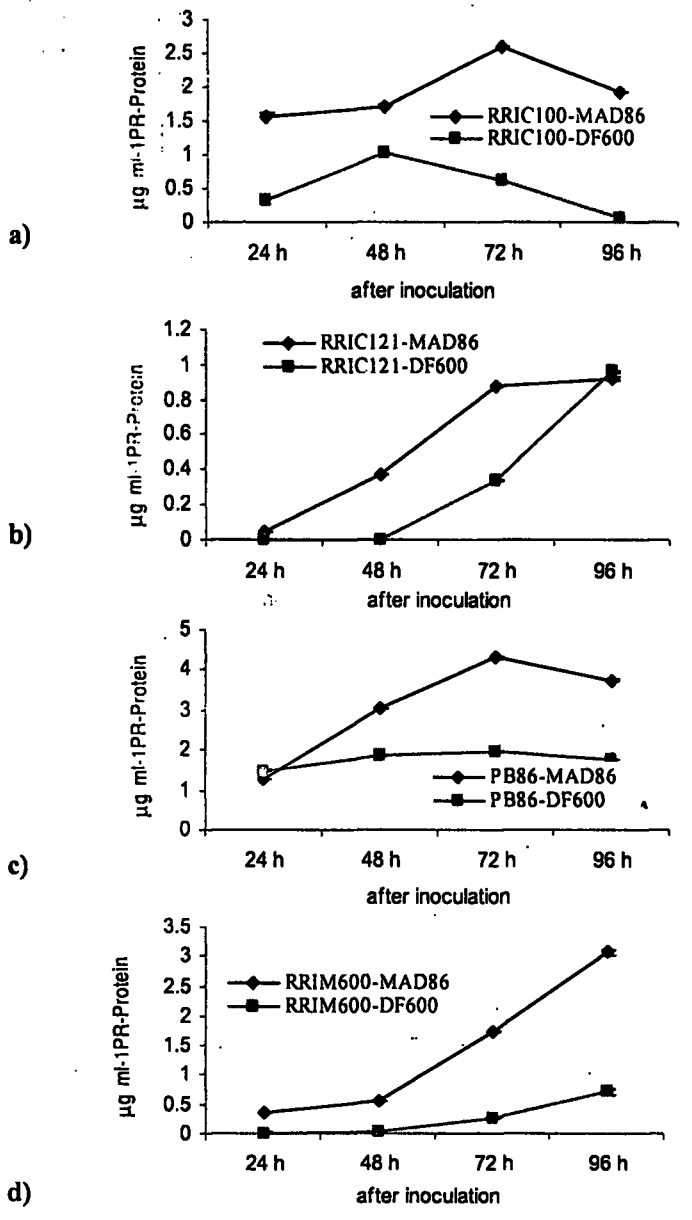
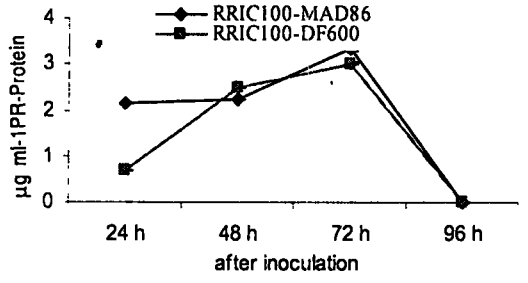
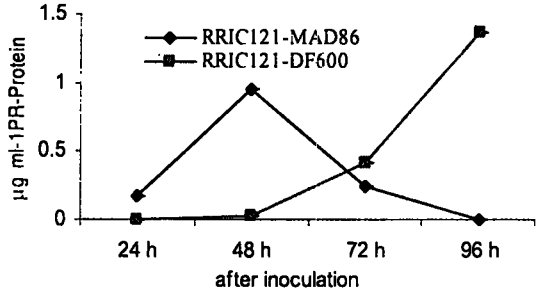


Fig. 1. a,b,c,d PR-protein concentrations formed *in vitro* (mg g^{-1} fresh weight of petioles) in petioles inoculated either with *P. meadii* MAD 86 or DF 600 strains in the laboratory (df=10, $P<0.05$). Bars represent SED (Standard error of deviation of 4 replicates)

e)



f)



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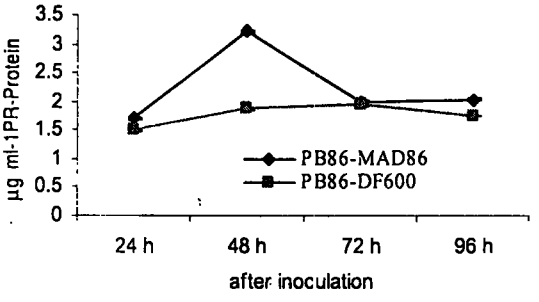

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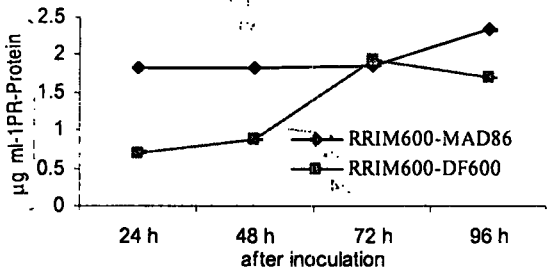

Fig. 1e,f,g,h. PR-protein concentrations formed in live attached petioles (*in planta*) inoculated similarly either with *P. meadii* MAD86 or DF600 strains (df=10, $P< 0.001$). Bars represent *SED* (standard error of deviation of 4 replicates).

infected with *P. meadii* strain MAD86 than that of infected with DF600. Healthy or *P. meadii*-infected petioles of RRIC100 had significantly ($P<0.001$) higher PAL activity up to 72 h period, while, in petioles of susceptible clones this was slightly different as there is a fall after 48 h in clones RRIC 121 and RRIM600. PAL activity in healthy petioles of each clone did not vary markedly (*SEM* values from three time points were low as 0.44-0.56) during 72 h period and therefore, they were separately averaged for comparison of PAL activity values of infected petioles at each time point (Table 1).

Discussion

Only five rubber genotypes were used in this investigation due to none availability of other genotypes, which are known as tolerant or susceptible to *Phytophthora* diseases. The responses of field plants to *Phytophthora* could be variable as such, leaves, stem or bark of same genotype can respond differently. However, in this study, susceptible genotypes had similar characteristics, which significantly varied from that of tolerant genotypes. Disease tolerance in rubber plants can be related due to both constitutive and induced substances, which may be detrimental to invading pathogens. Syntheses of β-1,3-glucanases and chitinases during pathogenesis have been implicated in the resistance of many patho-systems (Jebakumar *et al.*, 2001). Therefore, additional proteins (PR-protein) which are produced in petioles due to petiole infection by *P. meadii* have been measured. Higher PR-protein levels produced after 24 h in petioles of RRIC100 may have an impact on the infection, while low levels were produced in susceptible petioles which thereafter increased to higher levels upon infection. In this instance, it is difficult to consider PR-proteins as the only key substances involved in the host tolerance. However, studies showed that structural *b*-proteins are involved in resistance in tobacco which are the most acidic proteins of 15.5-15.8 kDa (Gianinazzi *et al.*, 1980).

Table 1. *Phenylalanin Ammonia Lyase (PAL) activity (mMol h^{-1} g^{-1}) in petioles of rubber genotypes upon infection by* Phytophthora meadii (IMI385259)

	Healthy petioles	Infected petioles		
Petioles		after 24 h	after 48 h	after 72 h
RRIC100	13.15 ± 0.56	28.03 ± 0.59 $^{(c)}$	30.73 ± 0.32 $^{(c)}$	32.97 ± 0.98 $^{(d)}$
RRIC121	11.76 ± 0.48	21.67 ± 0.38 $^{(b)}$	27.50 ± 0.21 $^{(a)}$	25.32 ± 0.64 $^{(a)}$
PB86	10.17 ± 0.54	25.24 ± 0.45 $^{(a)}$	27.56 ± 0.24 $^{(a)}$	30.69 ± 0.75 $^{(c)}$
RRIM600	08.87 ± 0.44	25.18 ± 0.66 $^{(a)}$	29.20 ± 0.36 $^{(b)}$	26.80 ± 1.11 $^{(b)}$

Values are means of 8 replicates ± SEM. Values in columns sharing same letters are not significantly different ($P<0.05$) according to the analysis of covariant used in proc glm in SAS program. The PAL activity levels of healthy petioles were used as the covariant.

PAL involves in producing phenolic compounds in plants *via* shikimic pathway. As a result of the higher activity of PAL in petioles of RRIC100, vanillin, which is toxic to *P. meadii* (Jayasuriya *et al.*, 2003), may have been produced initially. These phenolics may be important for tolerance, since the rate and the appearance of phenolic compound have been found directly related to the tolerance of rubber to *M. ulei* (Garcia *et al.*, 1995a; b). Low activity of PAL in susceptible petioles may have been related to production of sterols or triterpenes (Jayasuriya *et al.*, 2003), which may be less fungitoxic than vanillin. Lignin and cinnamic acid cause modifications in cell walls that may increase tolerance. Therefore, accumulation of PR-proteins synergistically with higher PAL activity may be considered as promising criteria to assess the tolerance of new rubber genotypes against *P. meadii* and these criteria should be tested against other pathogens too.

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Address for correspondence: Dr K E Jayasuriya, Department of Plant Pathology and Microbiology, Rubber Research Institute of Sri Lanka, Dartonfield, Agalawatta, Sri Lanka.
E-mail: kejayasu@sltnet.lk.
kithsiri.jayasuriya@yahoo.com