Measurements of Defense Responses as Criteria towards an Early Determination of Tolerance of *Hevea brasiliensis* Genotypes Against *Colletotrichum gloeosporioides*

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ABSTRACT

Responses of rubber against leaf infections caused by *Colletotrichum gloeosporioides* were investigated by measuring post-infectional activity levels of PAL(Phenylalanine ammonia lyase), peroxidases, accumulation of phenols and PR-proteins in surrounding areas of the infection. The increase of PAL enzyme activities in infected rubber leaves alone cannot be used as a criterion. Synergystical effect of post-infectional accumulated quantities of total phenolics along with increase of peroxidase and/or PAL enzymatic activity levels could be used to discriminate the tolerance or susceptibility of rubber clones against *C. gloeosporioides* infection.

KEYWORDS: Defense Reactions, PAL, Peroxidases, PR Proteins, Phenols, *Hevea brasiliensis*, *Colletotrichum gloeosporioides*

INTRODUCTION

The rubber [*Hevea brasiliensis* (Wild ex Adr. Juss) Muel Arg] is an economically important tree of family Euphorbiaceae, which provides latex containing natural rubber for rubber industry. It has a potential to provide dry rubber yield up to 2500 kg ha^{-1} yr^{-1} depending on the clone and other agronomic practices. The expected potential may not be achievable due to various factors such as poor quality of plants and grafting material used for propagation, unfavourable natural factors including leaf fall diseases and poor soil fertility *etc.* The low tree-stand per ha and weakened canopies due to diseases may be most significant factors attributed to the reduced yield and for low income from the industry.

Colletotrichum leaf disease is one of the main diseases of young rubber plantations including nursery plants. This has to be managed by application of hazardous chemicals, and therefore, the most suitable remedy is planting resistant genotypes in large scale plantations. However, rubber clones, which are tolerant to all diseases, are very few because rubber trees at any growth stages could be devastated by one or many fungal species or Oomycetes depending on the level of tolerance of the genotype (clone). Although, an immature plant of certain rubber genotypes such as RRIC100 is susceptible to leaf diseases in nurseries, later it tolerates most of leaf diseases in the field. On contrary, some genotypes such as RRIC103, RRIC110 or RRIC102, which were initially tolerant against *Corynespora* leaf fall disease caused by *Corynespora cassiicola* (Berk and Curt.), were later became vulnerable to the same disease largely. The high yielding genotypes so far produced to the rubber industry by the breeders have shown variable levels of tolerance to certain fungal pathogens depending not only on the basis of their parentage but also on agro-climatic conditions under which they were cultivated. Therefore, an early prediction about the disease tolerance of new rubber genotypes produced by the breeders is virtually impossible. However, screening of new rubber genotypes is done by conventional methods using plant nurseries established in different agro-climatic locations with the help of visual criteria determined by the plant pathologists. Results obtained from these screening methods may have some uncertainties since, many local environmental factors depending on location or altitude involve in disease incidences. Therefore, the early determination of the tolerance of rubber genotypes to the disease is important to minimize the expenditure incur on large scale experiments for screening purposes. Hence, it is important to identify main biochemical factors that are directly correlated with the disease tolerance in rubber tree.

Plant resistance is presumably be related to production of pathogenesis related proteins (PR-proteins) (Jebakumar *et al.*, 2001), higher activities of enzymes such as Phenylalanine ammonia lyase (PAL) (Yamamoto *et al.*, 1977) Polyphenol oxidases or peorxidases (Kombrink and Somssich, 1995). Nicole *et al.* (1985) has reported reactions of *H. brasiliensis* against root rot disease caused by *Rigidoporous lignosus* [presently *Rigidoporous microporus* (Fr.) Overeem] and *Phelinus noxius* (Corner) G.H.Cunn. Cellular hypertrophy and hyperplasia, cambium activity stimulation, lignification and suberification of certain walls were some rapid histological reactions they had observed. Nicole *et al.* (1991) reported an elicitation of root defense responses of *H. brasiliensis* to *R. lignosus* after the injection of fungal cell wall extract in to root system of 1 month old seedlings. Fifteen days after injection they have also observed stimulation of cinnamyl alcohol dehydrogenase activity, which involved in lignin synthesis and callose deposits.

Although such work has been carried out by various authors, they have not explored the resistance mechanism, merely to distinguish the resistant or the susceptible types. Therefore, this study was undertaken to explore the possibility of assessing the genetic resistance of some rubber genotypes against *C. gleoesporioides* using direct biochemical analysis such as rapid production of phenolics, activities of

peroxidases, PAL enzyme and PR-proteins in host leaf tissues during infection. Results are discussed in view of using these criteria for early determination of the tolerance of rubber clones against *C. gloeosporioides* leaf disease.

MATERIALS AND METHODS

Plant materials

Investigations have been carried out at the Department of Plant Pathology and Microbiology of the Rubber Research Institute, Agalawatte. Healthy immature leaflets (late copper brown stage with apple green appearance) were obtained from clones of RRIC100, RRIC130 which are tolerant to *C. gloeosporioides* and from BPM24, RRIC117, RRIC121 and PB235 as clones which are moderate to severely susceptible to *C. gloeosporioides* (Jayasinghe, 2000). Similarly, leaves of clones RRIC102 was also taken as samples that have not been established yet as either tolerant or susceptible.Semi-matured (late copper brown stage with apple green appearance) leaves were obtained from at least ten trees of each clone at each time for sampling purposes.

Isolation and maintenance of fungal cultures

C. gloeosporioides was isolated from naturally infected leaf tissues. They were cut in to small pieces, surface sterilized with 70 % ethanol for 2 min subsequently rinsing twice with sterilized distilled water. Thereafter, the tissues were aseptically transferred to potato dextrose agar (PDA-Difco) plates. The plates were incubated at room temperature (27±2°C) for 3 days in a glass culture chamber under normal light/dark conditions. Fungal hyphae that appeared to be *C. gloeosporioides* was transferred to fresh PDA plates and purified by repeatedly transferring. Purified cultures were maintained on PDA plates at 27±2°C in normal light/dark condition. A pure culture of *C. gloeosporioides* obtained from Plant Pathology and Microbiology Department of the Rubber Research Institute was also maintained in the similar manner.

Preparation of spore suspension

A standardized spore suspension was prepared by using 4-6 days old cultures of *C. gloeosporioides*. They were aseptically rinsed with sterilized distilled water, while gently scraping the mycelia with a sterile colour paint brush. Finally, the suspension was filtered through a sterile filter paper and diluted up to a concentration of 10^4 spores ml^{-1} using the haemocytometer.

Inoculation of healthy leaves

Healthy immature leaves obtained as described above was kept on moistened papers in petri plates as single leaf per plate.All Petri plates were kept in a plastic tray. The leaves were inoculated with the spore suspension and observed for the development of lesion. After 72 h of inoculation, the tissues were taken for investigations of enzymes activities. Healthy leaves, which were inoculated with sterilized distilled water, were served as control.

Comparison of PAL activity in infected and healthy leaves

The activity of PAL enzyme was determined as the rate of *trans*-cinnamic acid formed during the enzyme reaction (Bruesk, 1980). One gram of immature leaf tissue was extracted in 2 ml of ice-cold 0.1 M potassium phosphate buffer (pH 8.0) and filtered through two layers of muslin cloth. The filtrate was centrifuged at 7500*g* at 4°C for 20 minutes and the supernatant was used as a crude enzyme extract. The reaction mixture contained 0.1 ml of enzyme extract, 0.4 ml of 0.1 M borate buffer (pH 8.8) and 0.5 ml of 12 mM *L*-Phenylalanine. The reaction mixture was incubated for 1 h at 32°C. The reaction was terminated by addition of 1 M trichloroacetic acid. The absorbance was read at 290 nm using a UV/visible spectrophotometer (Camspec M330). The enzyme activity was expressed on fresh weight basis as *n*mol of *trans*-cinnamic acid released min^{-1} g^{-1} of leaf tissue.

Comparison of PR proteins in infected and healthy leaves

Two grams of leaf tissues obtained from the margin of infected lesions were gently homogenized and extracted with 3 ml of protein extraction buffer (pH 2.8) containing 84 mM citric acid, 32 mM Na_2HPO_4, 14 mM mercaptoethanol and 6 mM ascorbic acid. The extract was centrifuged at 15,340*g* for 30 min (Tuzan *et al.*, 1989), and the protein concentration of the supernatant was determined by Bradford (1976) with some modifications.

Protein concentration of each sample was determined by adding 100 μl of sample solution to 5 ml Coomassie blue dye reagent (100 mg Coomassie brilliant blue G-250 dissolved in 50 ml of 95 % ethanol to which 100 ml of phosphoric acid was added and diluted to 1 l with sterilized distilled water). In 10 minutes, the absorbance was measured with the aid of Camspec M330 at 595 nm.Protein concentration of the samples was calculated using the calibration curve plotted using a concentration gradient of Bovine Serum Albumin (Sigma). The difference between the protein concentration of healthy leaves and that of infected leaves was considered as additional protein concentration or PR-proteins that has been produced as a result of infection.

Comparison of Peroxidase enzyme activity of infected and healthy leaves

Five gram of infected and healthy leaf tissues were gently homogenized in 10 ml of ice-cold 0.2 M sodium phosphate buffer (pH 7.0). The homogenate was filtered through two layers of muslin cloth and then centrifuged at 15,000*g* for 20 minutes at 4°C (Canal *et al.*, 1988). The total protein content of the supernatant was determined according to Bradford (1976). Thereafter, the extract was dialysed overnight against 0.02 M sodium phosphate buffer (pH 7.0). The dialysed extract was assayed for peroxidase activity according to Rodriguez and Sanchez (1982). The assay

mixture contained 1.4 ml of 0.05 M phosphate citrate buffer (pH 4.6), 1 ml of 4 mM guaiacol and 0.5 ml of 26 mM H_2O_2. The mixture was incubated for 15 min at 25°C and finally 0.1 ml of the enzyme extract was added to the cuvette. Changes of absorbance at 420 nm were measured for 3 min using Camspec M330. Peroxidase activity was expressed as ΔA_{420} min^{-1} g^{-1} fresh weight.

Quantitative comparison of phenolic substances in infected and healthy leaves

One gram of healthy and infected leaf tissues of the above rubber clones was gently homogenized with 5 ml of boiling 80 % methanol. The homogenate was filtered through two layers of muslin cloth and thereafter, the methanol extract was evaporated to dryness. The residue was suspended in 2 ml of 80 % methanol and then centrifuged at 1000*g* for 5 minutes. Volumes of 0.1 ml of the supernatant, 0.5 ml of folin-ciocalteus reagent [100 g of sodium tungstate ($Na_2CuO_4.2H_2O$), 50 ml of phosphoric acid and 20 g of phosphomolibdic acid were added to 750 ml of distilled water and the mixture was refluxed for 2 h and diluted to 1 l] and 7.8 ml of distilled water was added to make the reaction mixture and allowed the mixture to stand for 3 min. One ml of Na_2CO_3 saturated solution was added and it was kept in room temperature for 1 h to complete the reaction. The absorbance was measured at 725 nm using Camspec M330. Phenol concentration was expressed as μg ml^{-1} g^{-1} fresh weight. Caffeic acid was used as the reference phenolic compound to draw the standard curve.

Statistical Analysis

Data groups have been analysed by General Linear Model using Genstat® Release 4.2 (2000) VSN international Ltd, Oxford, U.K.

RESULTS AND DISCUSSION

Phenylalanine ammonia-lyase activity of infected and healthy leaf tissues

The activity of PAL of the infected tissues of all clones are significantly ($p<0.05$) higher than that of the healthy tissues of same clones.However, the differences between each clone or between clones of two categories were not significant (Table1).

PR-proteins in infected and healthy leaves

According to the data of Table 2, in leaves of RRIC100 and RRIC102, highly significant quantities of PR-proteins were produced as a result of the infection by *C. gloeosporioides*, whereas in leaves of BPM24 and RRIC121, the amount of PR-proteins produced after infection were just above the limit of the significance. Protein level in infected tissues of RRIC 130 was significantly higher than that in the infected tissues of other clone. (Table 2)

Pre and post-infectional accumulation of phenolic substances in leaves

Phenol levels of the infected tissues were significantly higher than those of healthy tissues irrespective of the clone. However, this increase present in leaf tissues of tolerant clones was highly distinguishable than that in infected tissues of the moderate to severely susceptible clones (Table 3).

Peroxidase enzyme activity in infected and healthy leaves

The activity of peroxidase was significantly higher in the infected leaves of tolerant clones including RRIC102, which is not established as tolerant. A lower activity was observed in leaves of other clones, while in extracts of RRIC117, which was not significantly different with that of healthy tissues (Fig.1).

Table 1. PAL activities of the infected and healthy leaf tissues of rubber clones

Response to *C. gloeosporioides*	Clone	PAL mg ml^{-1} g^{-1} §			LSD
		Healthy	Infected	Increase	
Tolerant	RRIC 100	1.872^{f}	2.489^{d}	0.617 (32.9)	0.0147
	RRIC 130	2.225^{d}	2.671^{c}	0.446 (20.0)	0.0347
Moderate to severely susceptible	BPM 24	2.192^{e}	2.489^{d}	0.297 (13.5)	0.0138
	RRIC 121	2.258^{c}	2.814^{a}	0.556 (24.6)	0.0457
	PB 235	2.232^{d}	2.403^{e}	0.171 (7.7)	0.0209
	RRIC 117	2.427^{b}	2.735^{b}	0.308 (12.7)	0.0209
Sample clone	RRIC 102	2.743^{a}	2.825^{a}	0.082 (3.0)	0.0284

§ Values following same letters in the same column are significantly not different ($p<0.05$) according to the Duncan's Multiple Range Test (DMRT). Values in parentheses indicate percentages of the increases with respect to corresponding values of healthy tissues.

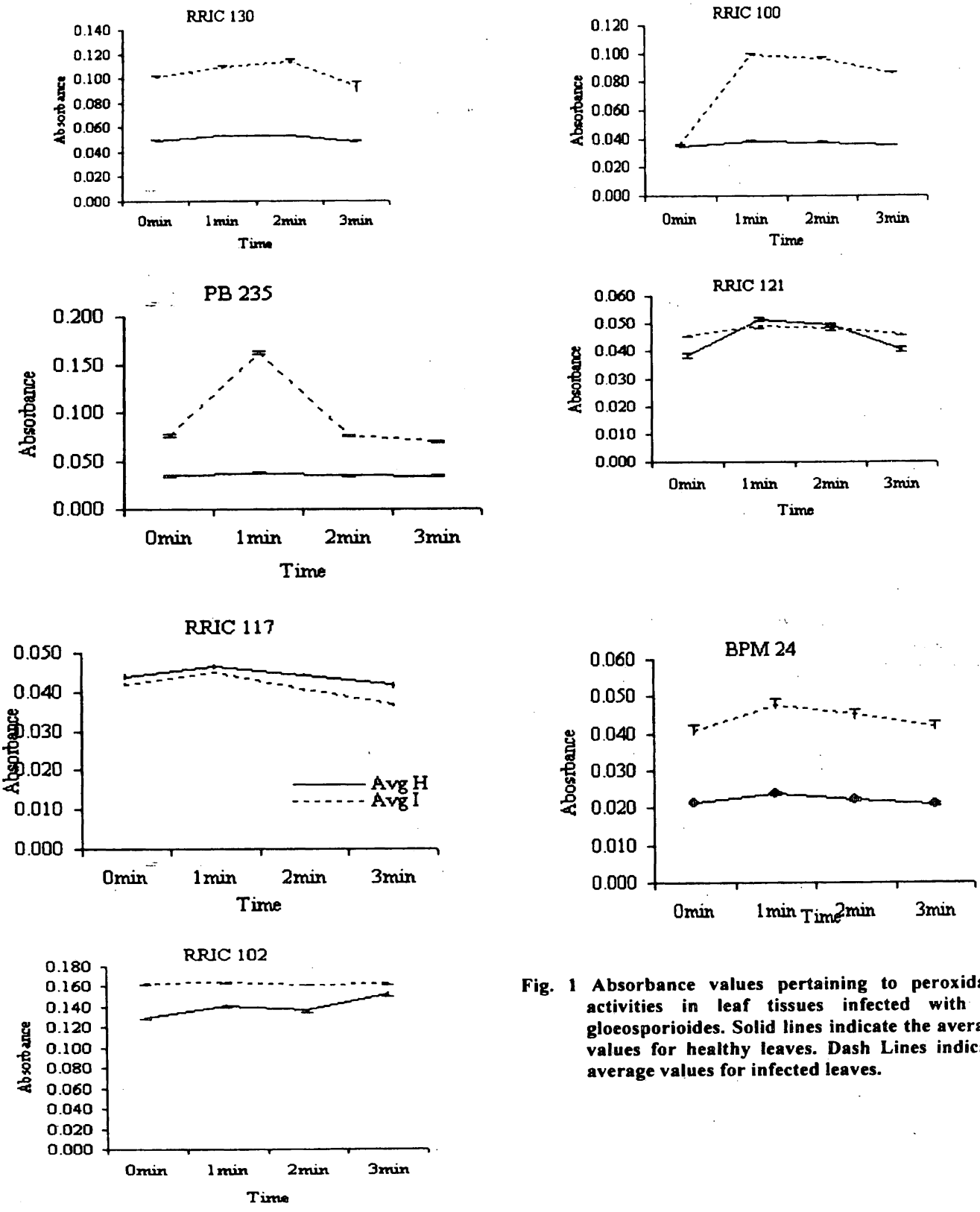

Fig. 1 Absorbance values pertaining to peroxidase activities in leaf tissues infected with C. gloeosporioides. Solid lines indicate the average values for healthy leaves. Dash Lines indicate average values for infected leaves.

Table 2. Protein level of the infected and healthy tissues of clones

Response to *C. gloeosporioides*	Clone	Protein mg ml^{-1} g^{-1}§			LSD
		Healthy	Infected	Increase	
Tolerant	RRIC 100	0.3284^{f}	0.3897^{e}	0.06 (18.3)	0.027
	RRIC 130	0.4235^{b}	1.0225^{a}	0.59 (139.3)	0.069
Moderate to severely susceptible	BPM 24	0.3495^{e}	0.3779^{f}	0.02 (5.7)	0.026
	RRIC 121	0.4613^{a}	0.6840^{b}	0.2 (43.3)	0.14
	PB 235	0.3304^{f}	0.3598^{g}	0.03 (9.07)	0.033
	RRIC 117	0.3767^{d}	0.4597^{d}	0.08 (21.2)	0.6
Sample clone	RRIC 102	0.3829^{c}	0.4709^{c}	0.09 (23.5)	0.032

§ Values with following same letters in the same column are significantly not different ($p<0.05$) according to the Duncan's Multiple Range Test (DMRT). Values in parentheses indicate percentages of the increases with respect to corresponding values of healthy tissues

Table 3. Levels of phenolic substances in infected and healthy tissues of rubber clones

Response to *C. gloeosporioides*	Clone	Phenols mg $ml^{-1}g^{-1}$			LSD
		Healthy	Infected	Increase	
Tolerant	RRIC 100	0.2771^{e}	0.6269^{b}	0.35 (126.0)	0.018
	RRIC 130	0.4815^{b}	0.6126^{b}	0.13 (27.0)	0.023
Moderate to severely susceptible	BPM 24	0.3697^{d}	0.3225^{b}	_	0.009
	RRIC 121	0.4364^{c}	0.5276^{b}	0.09 (0.21)	0.015
	PB 235	0.3700^{d}	0.3930^{b}	0.02 (0.05)	0.012
	RRIC 117	0.5061^{a}	0.5495^{b}	0.04 (0.08)	0.017
Sample clone	RRIC 102	0.4683^{b}	1.8020^{a}	1.33 (284.0)	0.15

§ Values following same letters in the same column are significantly not different ($p<0.05$) according to the Duncan's Multiple Range Test (DMRT). Values in parentheses indicate percentages of the increases with respect to corresponding values of healthy tissues.

PAL activated rapidly in the initial phase of the pathogenic invention on some plants (Kombrink *et al*, 1993) and catalysed the synthesis of lignin monomers and certain phytoalexins (Lamb *et al*, 1989). The extracts of infected leaves had higher activity of PAL than those of healthy leaflets. This agrees with the results obtained by Jayasuriya (2002), where a continuous increment of PAL activity was observed in petioles of RRIC100 and RRIC121 infected with *P. meadii*, during a period of 48 h. The highest increase in PAL activity in present work was found in leaves of tolerant RRIC100. However, increase of PAL activity between two clone categories showed no significant difference, indicating that a firm comparison using the PAL criterion only is difficult. Therefore, for discriminating different rubber clones on responses against *C. gloeosporioides*, use of PAL activity as a pre-screening criterion would be difficult. However, the higher PAL expression in the leaves of tolerant clones may be related to the accumulation of polyphenolics or lignin resisting fungal pathogens forming structural barriers (Jayasuriya, 2002).

Among the seven clones investigated, protein levels in infected tissues were always higher than those in healthy leaves. There was no correlation or significant differences between total protein contents in infected leaves of tolerant and moderate to severely susceptible clones. However, one cannot rule out the involvement of PR-protein in this case, since molecular masses of accumulated proteins were not characterized in the investigation.Even though the accumulated proteins were low in quantities, these proteins may be of low-molecular-weight structures enabling easy access through intracellular niches of leaf tissues. In addition, proteins accumulated largely in RRIC121 as a result of infection (43 % higher than in healthy leaves) may not be highly effective proteins (or β-glucanases), while the trace amount of proteins accumulated in infected leaves of RRIC100 or RRIC102 (18.3 % or 23.5 % respectively) may have higher impact on *C. gloeosporioides*. Most of the PR-proteins are responsible for thinning, degradation, swelling (hydrolysing) or lyses of pathogen cell wall or inhibition of the growth of pathogens (Kombrink *et al*, 1993). The production of such protein may vary in volumes or types apparently depending on the type of pathogen invaded.

Phenolic compounds in plants have been reported to be associated with the inhibitory effects against pathogens (Elmer *et al*, 1994). All tested clones have produced more phenolic substances upon infection by *C. gloeosporioides*. However, their increased accumulation was extremely higher in tolerant clones.Such phenolics in petioles of RRIC100 were reported to be highly fungi-toxic than those accumulated in susceptible clones (Jayasuriya *et al.*, 2003). Their absence or low rate of production may be an apparent reason for the susceptibility of BPM24, RRIC117, PB235 and RRIC121. Their enormous

production in leaves of RRIC102 as a result of infection can precisely be related to tolerance since peroxidases activity too compare well with the tolerant clones.Therefore, the sample clone RRIC102 can be designated as tolerant. This criterion may be used to differentiate tolerant and moderate to severely susceptible rubber clones against *C. gloeosporioides*.

The results show the active involvement of peroxidases in rubber leaves of tolerant clones to defend against *C. gloeosporioides*. This is possibly by production of toxic phenolic substances such as vanillin (Jayasuriya *et al.*, 2003) or related polymerized polyphenols such as lignin (Vance *et al.*, 1980; Fry, 1982). In addition, accumulation of PR proteins with increased activities of peroxidases may not be synergistically correlated with the tolerance level of some clones such as RRIC121 against *C. gloeosporioides*.It may be possibly because of not producing enough toxic phenolic substances or late production. These results may have been interpreted more precisely, if the tolerance level of used rubber clones had been determined more accurately in field conditions. The actual tolerant levels of some clones used in this study may be different from the present classification and the tolerant level may also change according to the conditions of the locality.

CONCLUSIONS

From all the facts, it may be possible to conclude that, for early determination of the tolerance of new rubber clones against *C. gloeosporioides*, the rate of the activity of peroxidases (OD_{420} 0.1 or above) in leaf extracts may be considered with the synergistic effect of the higher amount of accumulated phenolic substances (27-126 or above mg ml^{-1} g^{-1}) in laboratory conditions. The clone taken as a sample for observation, RRIC102, thus be designated as tolerant to *C. gloeosporioides*, since the analyzed data of the above reactions corresponded with that of the tolerant category.

ACKNOWLEDGEMENTS

Authors wish to thank Prof. D.P.S.T.G. Attanayake Head of the Department of Biotechnology, Wayamba University of Sri Lanka for his kind cooperation. The staff of the Plant Pathology and Microbiology Department of the Rubber Research Institute, Agalawatte, also deserve thanks for their fullest support extended throughout the investigation. Authors extend their thanks to Dr. (Mrs) Wasana Wijesuriya and her staff of the Biometry Department for their assistance in statistical analysis.

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