Jl Rubb. Res Inst Sri Lanka (1991) 71, 27-41

A STUDY OF ISOLATES OF *RIGIDIPORUS LIGNOSUS* THE CAUSATIVE AGENT OF WHITE ROOT DISEASE OF RUBBER

BY

R. L. C. Wijesundera,[1] S. P. Prelis

Department of Botany, University of Colombo, P. O. Box 1490, Colombo 3, SRI LANKA.

and

N. I. S. Liyanage[2]

Department of Plant Pathology, Rubber Research Institute, Dartonfield, Agalawatta, SRI LANKA.

ABSTRACT

Rigidiporus lignosus (Klotz.) Imazaki cause the white root disease of rubber *(Hevea brasiliensis).* Five isolates of the fungus, T1, 01, S1, F1 and H2, obtained from different rubber growing districts of Sri Lanka, were examined for their growth patterns in different solid media. The secretion of cell wall degrading enzymes was also studied in the isolates T1, S1 and H2 when grown in different media with different sources of carbon.

The isolate T1, which is known to be the most virulent, grew fast and densely on nutrient rich solid media such as potato dextrose agar (PDA) and malt extract agar (MEA), but its growth was poor on Czapek Dox agar, Knop-Glucose agar and the medium for wood rotting fungi The other four isolates also grew best on PDA and MEA, but the growth was less than that in T1. The growth of S1, O1, F1 and H2 on the other media however, was more than that of isolate T1,. All isolates except T1 formed branched, strand-like aggregations when growing on solid media.

Statistical analysis of the growth studies indicated that a significant difference existed among the isolates and also between the isolates and the media.

The isolate T1 secreted polygalacturonase, pectin lyase, B-glucosidase and cellobiase. The isolates S1 and H2 secreted only pectin lyase, B-glucosidase and cellobiase. In T1, the secretion of all the enzymes occurred earlier and the amount of enzyme secreted was

[1] Address for all correspondance.

[2] Presently, University of S. Pacific, Western Samoa.

also higher than in the other two isolates. No polygalacturonase', however, was detected when T1 was grown in the medium with the water insoluble component of rubber roots as the main source of carbon.

The pH for optimum activity of polygalacturonase was 6.5, while that for pectin lyase was 10.0.

INTRODUCTION

The white root disease caused by *Rigidiporus lignosus* (Klotz.) Imazaki is the most destructive disease affecting the rubber tree *Hevea brasiliensis* and is characterized by the production of a white rot in the butt-end of infected trees. The disease is of considerable economic importance in the plantations throughout the tropics including Sri Lanka. In Sri Lanka many isolates of *R. lignosus* with varying virulence have been obtained from different rubber growing regions (Liyanage *et al.*, 1977).

The fungus *R. lignosus* enters the roots of rubber tree either by direct penetration or via natural openings and during its growth in root tissue the fungus causes large scale tissue disruption (Peiris and Irugalbandara, 1973). The tissue disruption is thought to be due to the secretion of cell wall degrading enzymes by the fungus (Nicole *et al.*, 1982) and the tissue damage will ultimately destroy the entire root system thereby causing the death of the tree.

Most of the work on the white root disease in Sri Lanka has been confined to epidemiological aspects. Except for the work of Riggenbach (1960) no attempt has been made to study the physiological aspects of the disease.

Hence, the objective of this study was to compare the different isolates of *R. lignosus* with respect to their growth patterns and to assess their ability to secrete cell wall degrading enzymes under varying conditions.

MATERIALS AND METHODS

Organism

Five isolates of *Rigidiporus lignosus* namely T1, S1, O1, F2 and H2 were used in this study. All the isolates were obtained from the Plant Pathology department, Rubber Research Institute, Dartonfield, Agalawatta, Sri Lanka. Details of the isolates are given in Table 1.

Growth Media

The solid media used in the study were, potato dextrose agar (PDA), Czapek-Dox agar (CDA), malt extract agar (MEA), Knop-agar-glucose medium described by Riggenbach (1960) and a medium for wood rotting fungi (Liyanage *et al*,, 1977) having glucose 40 g, potassium dihydrogen phosphate 4 g, ammonium hydrogen phosphate 2 g, magnesium sulphate $7H_2O$ 2 g, calcium carbonate 0.2 g, calcium chloride 0.1 g, agar 15 g and distilled water 1 l.

The liquid medium described by Riggenbach (1960) was also used but without glucose and thiamine. Instead 10 g of either citrus pectin or sodium polypectate or carboxy methyl cellulose or the water insoluble component of fresh rubber roots were included in 1 l of the medium.

All media were sterilised by autoclaving for 20 min at 120°C, under a pressure of 15 lbs.

Preparation of water insoluble component of rubber roots.

Fifty g of ground fresh rubber roots (from clone PB 86) were macerated 3 times each in 100 ml of cold acetone (-5°C) for 10 min. The macerate was filtered through Whatman no. 1 filter paper using a Buchner funnel under suction, The filtrate was discarded. The residue was air dried and suspended in 150 ml of distilled water and washed by filtering as before. This was repeated till the filtrate was free of any reducing sugars. The filtrate was examined for the presence of reducing sugars by Nelson's (1944) modification of the Somogyi method.

Growth studies

Petri dishes of 9 cm diameter each having 20 ml of solid media were inoculated at the centre with plugs 0.5 cm diameter taken from the periphery of 6-day old cultures of each isolate of *R. lignosus* growing on MEA at 30°C. The inoculated plates were incubated at 30°C for a period of 5 days and growth assessed as described by Senaratna *et al.*, (1992). Five replicates were used in each experiment.

Secretion of cell wall degrading enzymes

Three isolates of *R. lignosus* differing in the type and rate of growth were selected for the study of secretion of cell wall degrading enzymes. The isolates selected were T1, H2 and S1.

Twenty ml of the modified Riggenbach liquid medium described above were dispensed in 100 ml Erlenmyer flasks and used to grow the fungal isolates for enzyme secretion studies. The medium in each flask was inoculated with two 0.5 cm diameter plugs taken from the periphery of 6-day old cultures of the isolates growing on MEA at 30°C. The inoculated media were incubated at 30°C without shaking. The cultures were harvested at 3-day intervals for a period of 27 days by filtering through Whatman no 1 filter paper. The pH, of the filtrates was measured immediately afterwards and the filtrates were used to test for cell wall enzyme degrading activity.

Enzyme assays

Polygalacturonase (PG: E.C. 3.2.1.15). The agar plate method (Dingle *et al.*, 1953) was used to determine PG activity. Activities were expressed relative to an aqueous solution (1.0 mg ml^{-1}) of pectinol 10M which was defined as having 100 units of PG activity ml^{-1}.

The release of reducing sugars from buffered solutions of polygalacturonic acid (Sigma Chemical Co.) was also used to determine PG activity (Wijesundera *et al.*, 1989).

Pectin lyase (PL: E.C. 4.2.2.10). PL was assayed and activity expressed as described in Wijesundera *et al.*, (1984).

Cellulose degrading enzymes. B–Glucosidase and cellobiase activity were measured by the hydrolysis of the chromogenic substances p-nitrophenyl o-B-glucopyranoside and p-nitrophenyl B-D-cellobioside respectively (Byrde & Fielding 1968). The reaction mixture consisted of 3.0 ml 0.1M sodium acetate buffer of pH 4.5, 0.5 ml aqueous solution of the substrate (1.0 mg ml^{-1}) and 1.5 ml of the test solution. After incubation at 30°C for 3h the reaction was terminated by adding 1.0 ml saturated Na_2Co_3 solution, and the colour of the phenate ion was estimated at 403nm. Necessary corrections were made for substrate and enzyme blanks and the activity was expressed arbitrarily as the absorbance over a unit period of time.

DISCUSSION

A significant difference existed in growth on solid media among the five isolates of the fungus used in this study. In addition the interaction between the isolates and the media was also significant. The isolate T1 which is very virulent (A. de S. Liyanage pers. comm.) grew very fast on nutrient rich media such as PDA and MEA, but on other media it exhibited very poor growth. T1 did not, at any time form branched strand-like aggregations like the other isolates which were examined. This agrees with the observations made in the soil where, even when adverse conditions are present T1 does not form strand-like aggregations or rhizomorphs (A. de S. Liyanage pers. comm.).

The growth of S1, O1, F2 and H2 was also slow on CDA, Knop-glucose agar and the medium for wood rotting fungi, but unlike in T1 very thin colonies were formed, the extension being by means of branched mycelial strands. Hence, one possible reason for the poor growth of T1 on media other than PDA and MEA may be its inability to form such branched mycelial strands.

The isolate T1 secreted PL, PG, B-glucosidase and cellobiase. The other two isolates examined secreted only PL, B-glucosidase and cellobiase. No PG was detected in cultures of S1 and H2 at any time. Isolate T1 always produced larger quantities of the enzymes and the enzymes were produced at earlier stages of growth than the other isolates.

In the medium with the water insoluble component of rubber roots PG was not detected even in cultures of isolate T1. However, the failure to detect the enzyme does not rule out the possibility that PG is secreted by the isolate in this medium. The non detection of PG in this medium could be due to the inhibition of the enzyme by the wall material such as wall bound proteins or the absorption of the enzyme by wall material or a combination of both factors (Cervone *et al.*, 1981; Jones *et al.*, 1972).

The pH optima obtained for both PG and PL of isolate T1 agrees with those reported for the two enzymes of several other plant pathogenic fungi (Cooper *et al.*, 1983). This therefore, serve as further confirmation of the secretion of both PL and PG.

Electron micrograph studies (Nicole *et al.*, 1982) have revealed that the destruction of the cell wall and middle lamella by *R. lignosus* is without doubt due to the action of cell wall degrading enzymes secreted by the pathogen. This implies a crucial role for these enzymes in the infection process. Hence, a large amount of enzymes at an early stage will result in a rapid and successful colonization of the host tissue. The isolate T1 is highly virulent, and its ability to produce PG, PL and also B-glucosidase and cellobiase in large quantities and at early stages of growth is a likely contributory factor in its high virulance (Byrde, 1979).

Formation of mycelial strands by T1 was not observed in this study and even in nature T1 is not known to form such structures. This may be related to the fact that when sufficient enzymes are secreted which will help to colonize plant tissues fairly easily the necessity of forming rhizomorph like strutures, which are means of growing over nutrient poor substrates, does not arise.

REFERENCES

Byrde, R. J. W. (1979). Role of polysaccharide degrading enzymes in microbial pathogenecity. In *Microbial polysaccharides and polysaccharases*, pp 417–736. Academic Press, London.

Byrde, R. J. W. & Fielding, A. H. (1968). Pectin-methyl-trans-eliminase as the maceration factor of *Sclerotinia fructigena* and its significance in brown rot of apple. *J. Gen. Microbiol.* 52: 287–297.

Cervone, F., Andebrhan, T., Coutts, R. H. A. & Wood, R. K. S. (1981) Effect of French bean tissue and leaf protoplasties on *C. lindemuthianum* polygalacturonase. *Phytopathol. Z.* 102, 238–246.

Cooper R. M. (1983). The mechanism and significance of enzymic degradation of host cell walls by parasites In *Biochemical Plant Pathology* pp 101–135. John Wiley & Sons Ltd., London.

Dingle, J., Reid, W. W. & Solomons, G. L. (1953). The enzymic degradation of pectin & other polysaccharides. II. Application of the cup plate assay to the estimation of enzymes. *J. Sci. Fd. Agric.* 4, 149–155.

Jones, T. M., Anderson, A. J. & Albersheim, P. (1972). Host-Pathogen Interactions IV. Studies on the polysaccharide degrading enzymes secreted by *F. oxysporum* f. sp. *lycopersici*. *Physiol. Plant Pathol.* 2:153–166.

Liyanage, G. W., Liyanage, A de S., Peiris, O. S. & Halangoda, L. (1977). Studies of the variability and pathogenecity of *Rigidiporus lignosus*. *J. Rubb. Res. Inst. Sri Lanka*, 54: 363–372.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153: 375–380.

Nicole, M., Geiger, J. P. & Nandris. D. (1982). Interactions hote–parasite entire *Hevea brasiliensis* et les agents de pourriture racinaire *Phellinus noxius* et *Rigidiporus lignosus:* Etude physio-pathologique comparee. *Phytopathol. Z.*, 105:311–326.

Peiris, O. S. & Irugalbandara, Z. E. (1973). Histology of *Hevea* roots infected by *Fomes lignosus*. *Ann. App. Biol.* 73: 1–7.

Riggenbach, A. (1960), On *Fomes lignosus* (Klotsch) Bres. the causative agent of the white root disease of the para rubber tree, *Hevea brasiliensis Muell.* Arg. *Phytopathol. Z.*. 40: 187–212.

Senaratna, L. N. K., Wijesundera, R. L. C. & Liyanage, A. de S. (1991).. Morphological and physiological characters of two isolates of *Colletotrichum gloeosporioides* from rubber *(Hevea brasiliensis) Mycol. Res.* 95: 1085–1089.

Wijesundera, R. L. C., Bailey, J. A. & Byrde, R.J.W. (1984). Production of pectin lyase by *Colletotrichum lindemuthianum* in culture and in infected bean *(Phaseolus vulgaris)*. *J. Gen. Microbiol.* 130: 285–290.

Table 1 The isolates of Rigidoporus lignosus *used in the study with the clones and the plantation districts from which they were isolated*

Isolate	*Estate*	*Division/District*	*Clone*
T1	Pitiyakande Estate	Pitiyakanda Division Kurunegala District	RRIC 41
O1	Padukka State Plantation	Main Division Colombo District	PB 86
S1	Peenakanda State Plantation	Parawatta Division Ratnapura District	PB 86
F2	Nakiyadeniya State Plantation	Digoda Division Galle District	PB 86
H2	Woodent Estate Ratnapura District	Rangegama Division	RRIC 100

Table 2 Growth of the isolates of Rigidoporus lignosus *on different media, measured as the mean diameter (cm) of colonies, on the 5th day after inoculation (Five replicates were used for each experiment)*

Media/Isolates	*T1*	*S1*	*O1*	*F2*	*H2*
Potato Dextrose Agar	8.7±0.64	7.4±0.43	5.2±0.25	6.2±0.69	4.6±0.25
Czapek-Dox Agar	0.4±0.10	2.8±0.57	2.2±0.54	3.1±0.43	1.4±0.63
Malt Extract Agar	5.7±0.30	2.7±0.32	2.6±0.25	2.5±0.25	1.6±0.26
Knop-Glucose Agar	0.0	2.3±0.3	1.3±0.25	2.8±0.25	2.3±0.75
Wood Rotting Medium	1.7±0.48	3.7±0.4	1.9±0.32	4.3±0.60	1.9±0.80

Table 3 The ANOVA table for the growth of the isolates of Rigidoporus lignosus *on solid media Growth was measured as the mean diameter of the colony and five replicates were used*

Source	*df*	*SSQ*	*MSQ*	*F Ratio*	*F Value*
Days	.. 8	764	15.28	6.846	2.220
Isolates	.. 4	268.6	67.15	30.085	2·378
Media	.. 4	1503.0	375.75	168.347	2.378
Isolate Media	.. 16	838.4	52.4	23.475	1.651
Isolate Day	.. 20	12.5	0.625	0.280	1.575
Media Day	..	20	31.5	1.575	0.705
Residual	.. 1430	3191.6	2.232		
Total	.. 1499	5921.5			

The difference between the isolates, among the media and the interaction between the isolates and media are significant at 95%.

Table 4 Secretion of pectin lyase (PL) by isolates of R. lignosus *when grown in Riggenbach liquid medium with different carbon sources*

Isolate	Carbon Source	Days after inoculation 3	6	9	12	15	18	21	24	27
		PL activity* in PL units/ml								
T 1 ..	Pectin	0.06 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.38 ± 0.06	0.21 ± 0.03	0.20 ± 0.02	0.08 ± 0.01	0.04 ± 0.01
	WIC	NA	NA	0.04 ± 0.01	0.15 ± 0.01	0.18 ± 0.03	0.12 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	NA
S 1 ..	Pectin	NA	NA	NA	0.04 ± 0.01	0.07 ± 0.02	0.12 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	NA
	WIC	NA	NA	NA	NA	0.05 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
H 2 ..	Pectin	NA	NA	NA	NA	0.05 ± 0.01	0.16 ± 0.01	0.03 ± 0.01	NA	NA
	WIC	NA	NA	0.04 ± 0.01	0.06 ± 0.02	0.09 ± 0.02	0.11 ± 0.03	0.05 ± 0.03	0.04 ± 0.01	NA

* Average of 8 replicates
NA— No Activity
WIC—Water insoluble component

Table 5 *Secretion of polygalacturonase (PG) isolate TI of* R. lignosus *when grow in Riggenbach liquid medium with different sources of carbon*

Days after inoculation	*PG Activity - PG Units**	
	Source of carbon	
	Pectin	*NaPP*
3	NA	NA
6	NA	NA
9	68±8	11±3
12	405±91	20±4
15	1721±201	55±11
18	370±53	669±50
21	47±12	343±40
24	55±11	9.0±2
2	NA	NA

* Average of 5 replicates
NA—No activity

Table 6 Secretion of B-glucosidase by isolates of R. lignosus *when grown in Riggenbach liquid medium with different carbon sources*

Isolate	Carbon Source	Days after inoculation 3	6	9	12	15	18	21	24	27
		B-Glucosidase activity-Relative units*								
T 1 ..	CMC	0.28±0.07	0.60±0.05	0.57±0.06	0.94±0.07	0.35±0.06	0.61±0.06	0.74±0.04	0.86±0.03	0.23±0.02
	WIC	0.20±0.04	0.74±0.06	0.48±0.05	0.46±0.06	0.64±0.03	1.01±0.08	0.72±0.06	0.84±0.02	0.24±0.03
S 1 ..	CMC	0.14±0.02	0.15±0.03	0.18±0.03	0.13±0.02	0.20±0.04	0.26±0.02	0.20±0.03	0.19±0.03	0.15±0.03
	WIC	0.08±0.01	0.10±0.01	0.09±0.01	0.08±0.01	0.07±0.01	0.12±0.01	0.10±0.02	0.08±0.02	0.06±0.01
H 2 ..	CMC	0.15±0.02	0.17±0.02	0.14±0.01	0.18±0.02	0.27±0.02	0.20±0.01	0.19±0.01	0.16±0.01	0.14±0.02
	WIC	0.07±0.01	0.12±0.02	0.08±0.01	0.10±0.01	0.15±0.02	0.14±0.02	0.17±0.02	0.15	0.12±0.02

*Average of 5 replicates
CMC—Carboxy methyl cellulose
WIC—Water insoluble component

Table 7 Secretion of cellobiase by isolates of R. lignosus *when grown in Riggenbach liquid medium with the WIC as the main carbon source*

Isolate	*Days after inoculation*								
	3	6	9	12	15	18	21	24	27
	*Cellobiase activity-Relative units**								
T 1	0.17 ± 0.01	0.70 ± 0.02	0.38 ± 0.04	0.36 ± 0.02	0.32 ± 0.02	0.26 ± 0.01	0.38 ± 0.02	0.32 ± 0.02	0.20 ± 0.04
S 1	NA	NA	0.0^4 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	01.3 ± 0.02	0.14 ± 0.02	0.21 ± 0.02	0.11 ± 0.02
H 2	0.06 ± 0.02	0.09 ± 0.01	0.11 ± 0.02	0.27 ± 0.02	0.20 ± 0.01	0.34 ± 0.01	0.52 ± 0.02	0.57 ± 0.03	0.22 ± 0.02

*Average of 5 replicates
NA—No Activity
WIC—Wa'er insoluble component