PROTEIN, ESTERASE AND PEROXIDASE PATTERNS OF PHYTOPHTHORA ISOLATES FROM COCOA IN MALAYSIA

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SUMMARY: Protein, esterase and peroxidase patterns of eighteen isolates of P. palmivora from various cocoa growing areas of Peninsular Malaya, Sabah dan Sarawak were studied by polyacrylamide vertical gel electrophoresis, to determine whether "strain" within P. palmivora could be distinguished using this method. The protein patterns of all the isolates studied were almost identical qualitatively and it was impossible to distinguish "strain" between the isolates of P. polmivora from cocoa. Esterase and peroxidase patterns of the isolates were also identical, although the amount of enzyme varies as observed by variation of color intensity of the bands.

Key Words: Phytophora palmivora; polyacrylamide vertical gel electrophoresis; protein, esterase; peroxidase.

INTRODUCTION

Phytotphthora isolates which causes black pod and canker disease of cocoa formerly asigned to P. palmivora (Butl.) Butl., has recently been recognized as belonging to at least two distinct species, P. palmivora and P. megakarya, on the basis of morphological form and chromosome size and number (2). A third species, Phytophthora Mf 4 has also been identified from cocoa closely related to P. capisici. As in other groups of fungi, classification is predominantly based upon morphological and cultural characteristics. Morphological variability of these structures, produced under the influence of different environmental and nutritional conditions, compounds the problem. Recently several noter researches recognised the usefulness of biochemical method as an aid in identification of Phytophthora species (3,6,8). But such an approach has been rarely used to distinguish pathogenic "strain" within species of the same morphological forms, although Koosiri *et al.* (9) has reported the possible use of polyacrylamide-disc-gel electrophoresis of protein, esterase and peroxidase as a tool in characterizing the morphological forms of P. palmivora isolates from cocoa. The present investigation was initiated to determine whether strain variations with in P. palmivora isolates from cocoa in Malaysia could be distinguished by their protein, esterase and peroxidase patterns separated by polyacrylamide vertical gel electrophoresis methods.

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MATERIALS AND METHODS

Eighteen isolates of P. palmivora originally isolated from cocoa pods from different cocoa growing areas in Peninsular Malaya, Sabah and Sarawak were used. Information on the code, place of origin and hosts of the isolates is presented in Table 1.

Single-spore culture of the isolates were maintained on corn meal agar (CMA), Five 5 mm plugs taken from the advancing edge of 4 day-old CMA cultures were transferred to 50 ml clarified V-8 juice broth in 250 ml Erlenmeyer Flasks and cultures were incubated in the dark at 25°C for 7 days in standing culture. Extracts were prepared from homogenised 0.2 g freeze dried Mycelium in 5 ml cold 0.1 M potassium phosphate buffer. The protein content of the extract was determined by Lowry's Folin test (10). Protein concentration in each sample was adjusted to about 200 μ/ml extract before electrophoresis was carried out.

Table 1: Isolates of Phytopthora palmivora isolated from cocoa pods used in the study.

Cod name of isolates	Origin	Host
AB 3	Bleinheim Estate, Perak	Theobroma cocoa
ABB 76	Bukit Belimbing, Perak	"
AF 4	Flemington Estate, Perak	"
AF 5	Flemington Estate, Perak	"
AJ 5	Jenderate Estate, Perak	"
AKB 4	Kuale Bernam, Selangor	"
BAU	Bau, Sarawak	"
BUY	Ulu Yam, Selangor	"
CST 1	Sungai Tekam, Pahang	"
JM 2	Mersing, Johore	"
JS 1	Sagil, Johore	"
KTB	Padang Serai, Kedah	"
LD	Lahad Datu, Sabah	"
MCE 79	Craigilea Estate, Malacca	"
MD 76	Devon Estate, Malacca	"
MM 78	Merlimau, Malacca	"
TRT	Tarat, Sarawak	"
TWU	Tawau, Sabah	"

Vertical electrophoresis of the buffer soluble protein was performed on 7.5% SDS-polyacrylamide gels, whereas for peroxidases and esterases sample extracts were loaded onto 7.5% polyacrylamide gels. Electrophoresis was carried out in a LKB vertical electrophoresis apparatus at 10°C, using tris glycine buffer at pH 8.3. Samples of isolates were run in duplicates and replicated twice.

For total soluble protein the gels were stained in Coomassie Brilliant Blue R250 for 2hr. and destained by rinsing with several changes of destaining solution. Nonspecific esterase activity was detected by immersing the gels in a freshly prepared staining solution made up of 0.1g of α-naphtyl acetate and 0.12 of β-naphtyl acetate dissolved in 10 ml of 70% ethanol; 0.5 g fast blue RR; 4 ml of propanol and 60 ml of phosphate buffer. Peroxidase activity was detected by floading the gels with 0.1% aqueous phyrogallol solution for 15 mins and then with 0.4% aqueous hydrogen peroxide.

Protein, esterase and peroxidase patterns were evaluated on the basis of number, position, intensity and Rf values. The Rf values were calculated by expressing the mobility or relative position of each band in relations to the tracking dye. Protein bnads were considered similar when there was more than 50% overlap. An index of similarity between the isolates was then calculated according to the formula:

$$\frac{\text{Number of bands in common x 2}}{\text{Total number of bands}} \text{ x 100}$$

Figure 1: Diagrammatic interpretations of the electrophoretic separations of isolates of Phytophthora palmivora from cocoa.

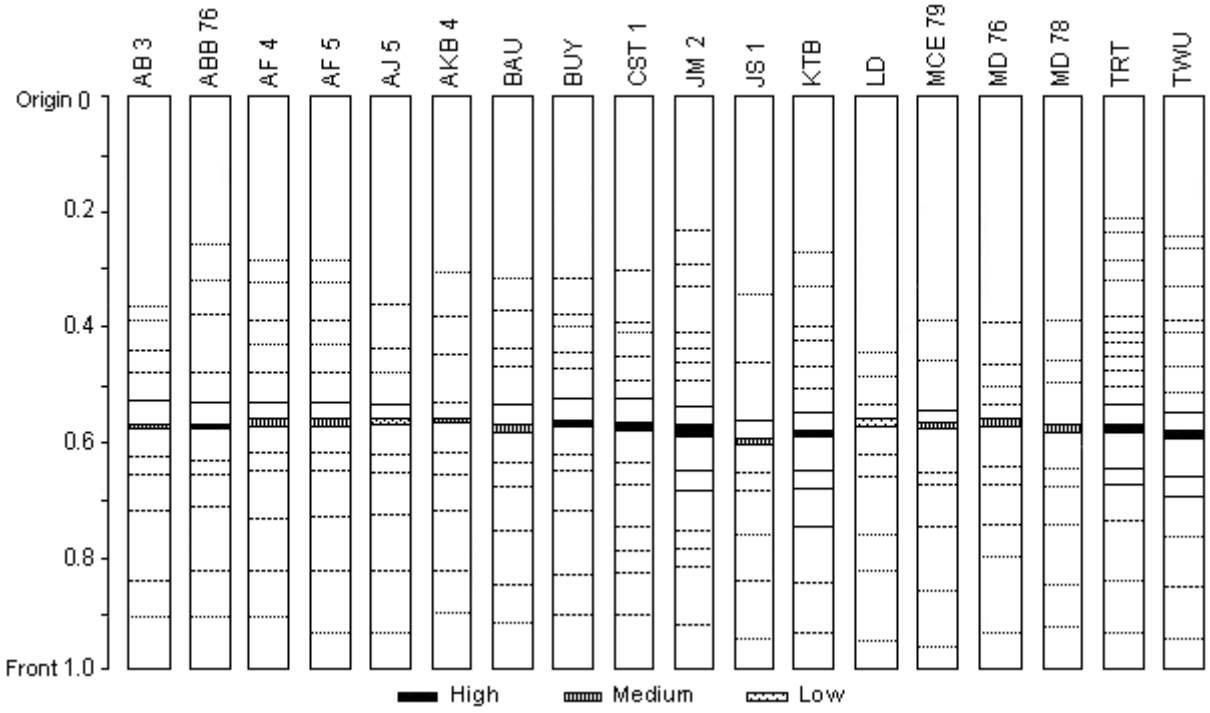


Figure 2: Percentage of similarity of protein patterns of of Phytophthora palmivora isolates from cocoa.

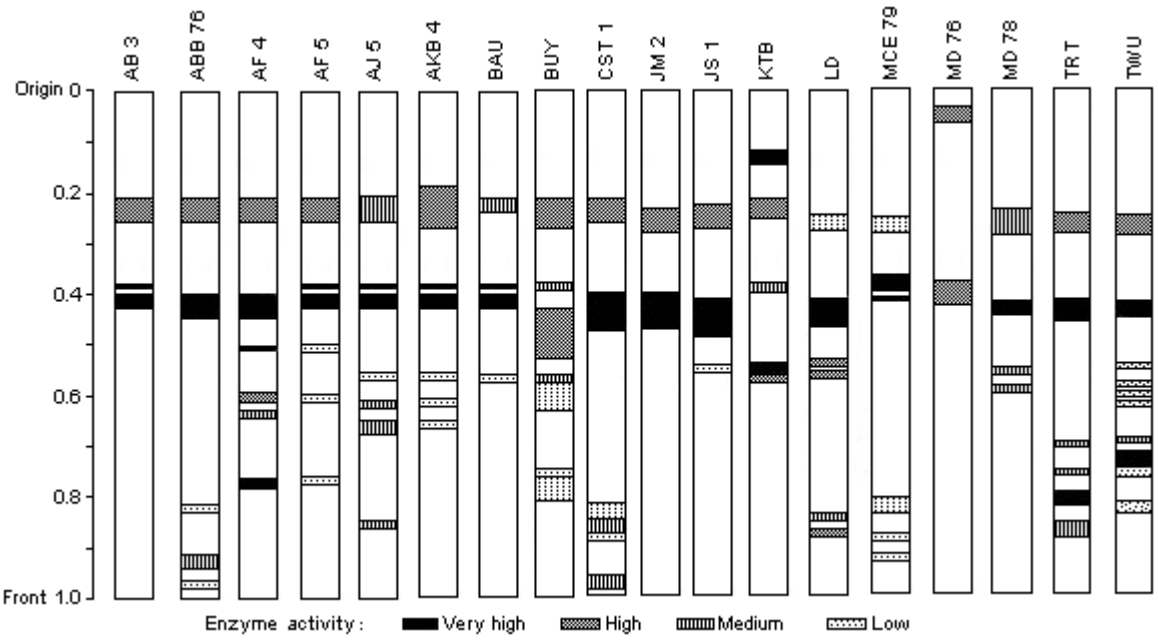
ISO-LATE	AB3	ABB76	AF4	AF5	AJ5	AKB4	BAU	BUY	CST1	JM2	JS1	KTB	LD	MCE79	MD76	MM78	TRT	TWU
ABB 76	73																	
AF4	70	61																
AF5	70	61																
AJ5	76	57	73	73														
AKB4	48	48	73	73	70													
BAU	73	46	44	44	57	48												
BUY	78	61	83	83	64	90	52											
CST1	50	50	48	48	17	61	44	56										
JM2	39	46	59	59	40	32	62	22	56									
JS1	30	20	38	38	21	21	40	57	86	50								
KTB	42	42	40	40	35	17	52	48	39	64	64							
LD	60	40	57	57	53	53	40	38	46	50	56	27						
MCE79	30	40	29	29	32	32	30	67	64	58	44	55	44					
MD76	40	50	67	67	21	21	60	48	55	58	67	73	56	78				
MM78	40	50	38	38	21	21	70	48	81	58	56	81	33	67	89			
TRT	29	56	76	76	44	37	64	69	33	75	54	80	39	46	77	62		
TWU	32	48	40	40	33	25	48	46	37	62	70	89	35	52	52	44	71	

RESULTS AND DISCUSSION

The reporducibility of electrophoretic pattern of each isolate examined was found to be identifical regardless of whether the extract was fresh or frozen, reproducibility of an isolate was also identical to that of a different culture of the same isolate grown at different time.

The protein pattern of all isolates of Phytophthora studied were closely identical qualitatively regardles of the locality from which the isolate was collected, but quantitative variation in the relative amounts of various proteins present were common as judged by the various thickness and color intensity of bands of similar Rf values (Figure 1).

Figure 3: Diagrammatic repesentation of electrophoretic esterase patterns of isolates of Phytophthora palmivora from cocoa.



In additions to the common band there was a wide diversity in number, position and witdth of very faint or light bands. All the protein bands were detected between Rf values of 0.2 to 0.95. As was emphasized by Boccas, *et al.* (1), the fact that only a portion of the total protein is analysed in these techniques could limit the sensitivity of such comparisons. Also, each solid band may be a mixture of different proteins of more or less identical mobilities. Thus, it is possible that the protein fractions responsible for pathogenicity on cocoa differentials may be present in too small a quantity to be detected by this technique. Further, the lack of differentiation of strains of Phytophthora isolates from cocoa by this technique could be attributed to the possibility that strains are the result of a response of the host to this fungus or of possible in vivo enzymatic differences after the host pathogen establishment, as suggested by Stavely *et al.* (14).

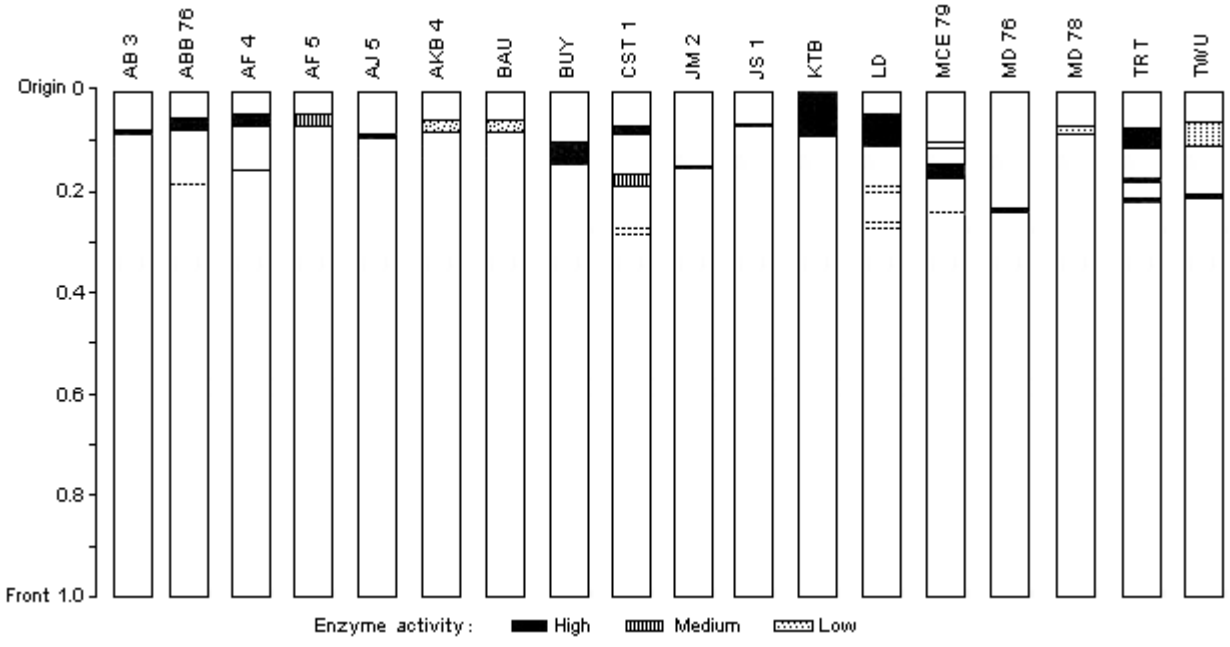
The index of similarity between gross protein patterns of the isolates was found to be high, in the range of 50-80% (Figure 2). It was also noticed that the differences between isolates of Phytophthora was not greater than those observed between replicates within individual isolates. These differences could be due to slight changes in the sensitive polypeptides during extraction.

Non-specific intracellular esterases have been studied by electrophoresis as an additional method for distinguishing species of fungi in the genera Aspergillus (4) and Phytopthora (8). But, this technique was not helpful in the separation of Verticillium dahlia Kleb. and V. albo-atrum Reinke and Berth or of isolates within forma of Fisarium oxysporum Schlect. Emend. Snyr: and Hans. (11). However, this studies showed that the distribution sites of esterase activity of all isolates of Phytophthora from cocoa were very similar. Major common bands were detected within Rf values of 0.2-0.6. Although the general patterns of esterase isozymes were similar in all the isolates studied, the amount of enzyme present varies between the isolates as observed by variation of color intensity of the bands (Figure 3) Hall *et al.* (8) used horizontal polyacrylamide gels and found three esterase patterns in P. cinnamomi, one in P. cactorum and nine in P. palmivora.

Peroxidase activity has not been much investigated by electrophoresis in Phytophthora species. In higher plants peroxidases are believed to be associated with indoleacetic-acid (IAA) oxidation (5), but in fungi their functions are not known. This investigation indicates that buffer soluble peroxidases extracted from mycelia of isolates of Phytophthora from cocoa can be detected by vertical polyacrylamide gel electrophoresis. At least one major and one to two minor sites of peroxidase activities were detected from each isolate (Figure 4) but no significant difference were noted in the position and Rf values.

Data presented here had shown that protein, esterase and peroxidase patterns could not show strain variations within isolates of Phytophthora from cocoa in Malaysia.

Figure 4: Diagrammatic presention of the electrophoretic patterns of peroxidases of isolates of Phytophthora palmivora from cocoa.



Earlier studies using detached pod inoculations had demonstrated variations in the degree of virulence of the isolates (13). Therefore according to the conditions of the present test it may be concluded that electrophoretic patterns of protein, esterase and peroxidase cannot be used to distinguish between strains within species, although such patterns have been found to be a useful tool level by previous workers. However, improved techniques may provide better separation and resolution for distinguishing differences in protein, esterase and peroxidase patterns.

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