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Electrophoretic protein patterns of three species of *Phytophthora* associated with black pod disease of cocoa (*Theobroma cacao* L.)

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Abstract. When electrophoretic profiles of native proteins from vegetative mycelia of *Phytophthora palmivora*, *Phytophthora capsici* and *Phytophthora citrophthora* causing black pod disease of cocoa in India were compared on a single polyacrylamide gel, the isolates of same species were readily distinguished both qualitatively by visual similarity in banding patterns and quantitatively by calculating similarity coefficients. Similarity coefficients were generally much higher between isolates within a species than between isolates of different species. The dendrograms obtained after unweighted pair grouping with arithmetic averaging cluster analysis, revealed that all the isolates of *Phytophthora capsici* were highly homogenous and formed a single cluster. The isolates of *Phytophthora citrophthora* were resolved into two electrophoretic types which were clustered into two distinct sub groups. *Phytophthora palmivora* formed a separate group. Thus, the results reveal that polyacrylamide gel electrophoresis can be used successfully in distinguishing species and sub groups within a species of *Phytophthora* encountered on cocoa.

Keywords. *Phytophthora palmivora*; *Phytophthora capsici*; *Phytophthora citrophthora*; electrophoretic protein profiles.

1. Introduction

Black pod disease of cocoa (*Theobroma cacao* L.) is a serious problem of economic importance to cocoa growers in India. Recent detailed studies on taxonomic complex of *Phytophthora* causing black pod disease of cocoa revealed the occurrence of *P. almivora* (Chowdappa and Chandramohanan 1993), *P. capsici* (Chowdappa *et al* 1993) and *P. citrophthora* (P Chowdappa and R Chandramohanan, unpublished) in India. These *Phytophthora* species have wide host range and also cause diseases of many tropical plantation crops. Further more, all these species of *Phytophthora* cause similar symptoms on cocoa and also exhibit wide variability in morphology within a species.

The identification of the *Phytophthora* species encountered on cocoa was mainly based on cultural and morphological criteria (Stamps *et al* 1990). Variability in morphology within and between species of *Phytophthora* was often found to be too large to allow accurate identification and thus lead to description of "atypical" isolates, which were later assigned to a different species (Brasier and Griffin 1979). In addition to morphology, the number and size of chromosomes have also been

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used to detect variation between species of *Phytophthora* on cocoa (Brasier and Griffin 1979). Identification of *Phytophthora* spp. based on these characters requires considerable time and experience. While morphology may continue to be important in the identification of *Phytophthora* species of cocoa, simple and less ambiguous criteria would be helpful in identification. Electrophoresis of soluble proteins from mycelia has been proved as an useful aid in identification and classification of various *Phytophthora* species (Kaosiri and Zentmyer 1980; Erselius and de vallavieille 1984; de vallavieille and Erselius 1984; Hansen *et al* 1986; Bielenin *et al* 1988). The objective of the present study was to compare protein profiles of isolates belonging to *P. palmivora*, *P. capsici* and *P. citrophthora* causing black pod disease of cocoa in India and to examine the utility of protein electrophoresis as an additional tool in identification.

2. Materials and methods

Acrylamide, N,N^1-methylene bis acrylamide, ammonium persulphate and bovine serum albumin (BSA) were purchased from Sigma (USA). All other chemicals used were of analytical reagent grade.

2.1 *Isolates*

Eleven selected isolates belonging to *P. palmivora*, *P. capsici* and *P. citrophthora* were examined. One isolate of *P. palmivora* (I-CP/122) from Vittal, Dakshina Kannada district, where the present investigation was carried out, was selected as there was no wide variation in cultural and morphological characters among the isolates of *P. palmivora* occurring in India and also *P. palmivora* has been found to be highly homogenous species based on isozyme analysis (Oudemans and Coffey 1991). Five isolates of each species of *P. capsici* and *P. citrophthora* were included in the present study as the isolates of *P. capsici* and *P. citrophthora* were limited in number in the population of *Phytophthora* occurring in India and they were reported recently as the causal organism of black pod disease in this country. It has also been reported that there were sub-group within each species of *P. capsici* and *P. citrophthora* on the basis of isozyme analysis (Oudemans and Coffey 1991) and mitochondrial DNA polymorphism (Förster *et al* 1990). Geographic origin and other details of the isolates used in this study are presented in table 1.

2.2 *Culture conditions*

The mycelium was grown in a defined liquid glucose-asparagine medium (Gill and Zentmyer 1978) without yeast extract and peptone. Isolates were grown in 500 ml conical flasks containing 100 ml of liquid glucose-asparagine medium. Each flask was inoculated with five mycelial disks, each of five mm diam., cut from the advancing margin of three-day-old cultures grown on carrot agar (CA) in dark. The inoculated flasks were incubated in dark at 24 ± 1°C for seven days. Then, the mycelial mat was harvested by filtering through Whatman No. 1 filter paper, washed with phosphate buffer (pH 7·0) and damp dried.

Table 1. Origin of isolates of *Phytophthora* spp. used in the present study.

Isolate No.	Geographic origin (Dist.)	Cropping systems
P. palmivora		
I-CP/122	Vittal, DK	Arecanut and cocoa
P. capsici		
I-CP/23	Devikulam, Idukki	Pepper, cardamom and cocoa
I-CP/25	Udumbanchola, Idukki	Pepper and cocoa
I-CP/27	Udumbanchola, Idukki	Coconut, pepper and cocoa
I-CP/76	Ranni, Pathanamthitta	Coconut and cocoa
I-CP/89	Kannara, Thrissur	Coconut and cocoa
P. citrophthora		
I-CP/26	Udumbanchola, Idukki	Coconut, arecanut, cocoa and coffee
I-CP/75	Thiruvalla, Pathanamthitta	Coconut and cocoa
I-CP/92	Thrissur, Thrissur	Rubber and cocoa
I-CP/93	Thrissur, Thrissur	Arecanut and cocoa
I-CP/96	Thrissur, Thrissur	Coconut, cocoa and pepper

2.3 *Extraction of soluble proteins*

The buffer-soluble proteins were extracted by grinding 2 g of damp-dried mycelium with a pestle and mortar containing acid-washed sand (0·5 g) and 1 ml of 0·1 M phosphate buffer (pH 7·0). The mixture was centrifuged at 12,000 *g* for 45 min. The resultant clear supernatant liquid from the fungal extract was decanted and immediately used for electrophoresis. All the operations were carried out at 4°C.

2.4 *Protein estimation*

Protein concentration was determined by the method of Lowry *et al* (1951) using BSA as standard.

2.5 *Electrophoretic separation of native proteins*

Electrophoresis of native protein preparations was carried out on a discontinuous system using 2.5% stacking gel and 7·5% separating gel in a vertical slab of $30 \times 22 \times 10$ cm size according to the method of Laemmli (1970). Gels were fixed, stained and destained according to Bielenin *et al* (1988). Electrophoresis buffer was Tris-glycine buffer (pH 8·3). Sample loading buffer was mixed with soluble protein preparations in the ratio of 1 : 1 and aliquots containing 80 µg of proteins of each *Phytophathora* isolate were placed into well of the gel. Electrophoresis was performed at 60 V for stacking gel and at 120 V for separating gel at 4°C. The protein patterns were visualized by staining the gels for 7 h with Coomassie brilliant blue G in water : methanol : perchloric acid (15 : 1 : 4) mixture and destained with several changes of mixture of water : methanol : acetic acid (7 : 2 :1).

2.6 *Similarity coefficients*

Similarity coefficients were determined for all possible pairs of *Phytophthora* isolates (Hansen *et al* 1986). For this, stained gels were cut to separate the lanes and comparisons were made by aligning the gels side by side. Gels were compared using two state characters for the presence or absence of protein bands. The total number of protein bands of any two isolates and the number of bands in common were recorded. Similarity coefficients were calculated using the following formula

$$2 \times \frac{\text{Bands in common}}{\text{Bands in isolate A} \times \text{Bands in isolate B}} \times 100.$$

2.7 *Unweighted pair grouping with arithmetic averaging cluster analysis*

Unweighted pair grouping with arithmetic averaging (UPGMA) was used to construct dendrograms from similarity coefficients (Sneath and Sokal 1973).

3. Results

When the electrophoretic profiles of native proteins of isolates of *P. palmivora*, *P. capsici* and *P. citrophthora* were compared on a single gel, the three species could be readily distinguished both qualitatively by visual similarity in banding patterns (figure 1) and quantitatively by calculating similarity coefficients (table 2). A total of 21 protein bands were observed on the gels, although no single isolate possessed all of these. The protein banding patterns of *P. palmivora* differed by 15 bands from the patterns for isolates of *P. capsici* and 13 bands from the patterns for isolates of *P. citrophthora*. The protein banding pattern of *P. capsici* differed by 15 bands from the pattern for isolates of *P. citrophthora*.

Similarity coefficients were generally much higher between isolates within a species than between isolates of different species (table 2). Similarity coefficients between *P. palmivora* and *P. capsici*, *P. citrophthora* and *P. palmivora* and *P. capsici* and *P. citrophthora* were 25, 54 and 30·8 to 41·2% respectively. The dendrograms obtained after UPGMA cluster analysis revealed that all the five isolates of *P. capsici* were highly homogenous and formed a single cluster (figure 2). The five isolates of *P. citrophthora* were resolved into two electrophoretic types which were clustered into two distinct subgroups. Similarity coefficients between two groups was 64·7%. *P. palmivora* formed a separate group.

4. Discussion

Several characteristics, including protein profiles, have been proposed (Brasier 1983; Gallegly 1983) to supplement morphological characters that were being used as sole determinants for identification and classification of *Phytophthora* species. The validity of protein banding patterns as a major determinant for distinguishing species and subgroups within a species of various *Phytophthora* species was highlighted by various workers (Kaosiri and Zentmyer 1980; Erselius and de vallavieille 1984; de vallavieille and Erselius 1984; Hansen *et al* 1986; Bielenin *et al* 1988). Using

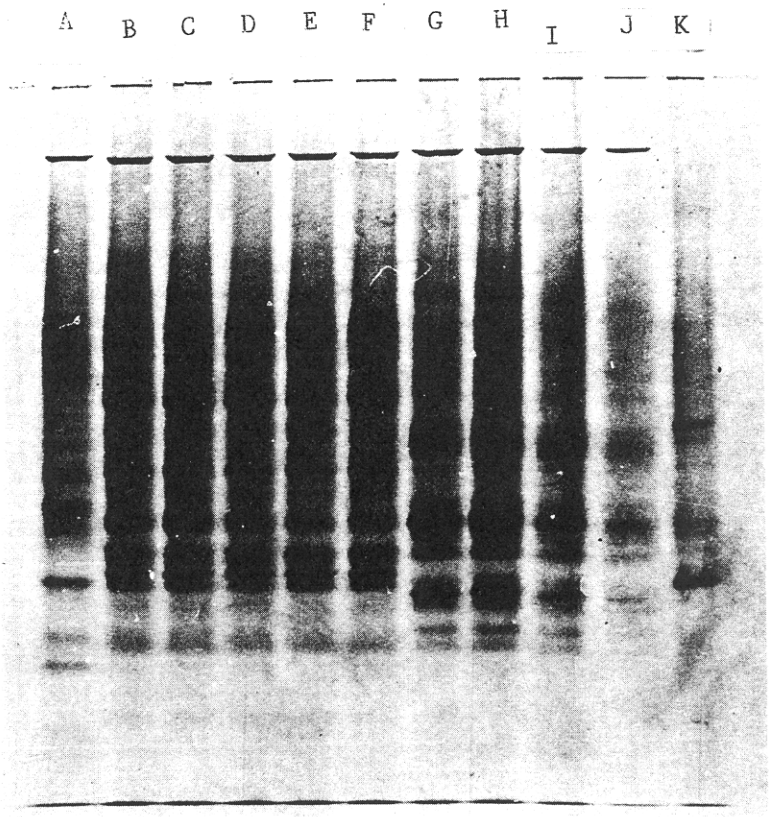

Figure 1. Protein profiles for three species of *Phytophthora* as differentiated by electrophoresis of native proteins. (**A**), I-CP/122; (**B**), I-CP/23; (**C**), I-CP/25; (**D**), I-CP/27; (**E**), I-CP/76; (**F**), I-CP/89; (**G**), I-CP/26; (**H**), I-CP/92; (**I**), I-CP/93; (**J**), I-CP/96; (**K**), I-CP/75.

electrophoretic protein banding patterns alone, Hamm and Hansen (1983) described *P. pseudotsugae* as a new species of *Phytophthora*. Also, in support of electrophoretic techniques as a functional taxonomic criterion protein profiles were employed as major criterion for distinguishing subgroups of *P. megasperma* (Hansen *et al* 1986). Further, electrophoretic protein profiles were found to be distinct for each species of *Phytophthora* regardless of isolation date, geographic locality, age of the mycelium, growth medium (either natural or synthetic with varying carbon and nitrogen sources), culture conditions or virulence race (Gill and Zentmyer 1978; Kaosiri and Zentmyer 1980; Xu *et al* 1982; Hansen *et al* 1986; Bielenin *et al* 1988; Guncu and Cinar 1989; Wilcox *et al* 1993). In the present study, polyacrylamide gel electrophoresis of soluble mycelial proteins provided a reproducible and sensitive finger print for *P. palmivora*, *P. capsici* and *P. citrophthora*. Isolates of these three species could be visually distinguished on the basis of contrasting native protein banding patterns whereas isolates within a single species produced largely homogenous banding patterns. UPGMA cluster analysis based on similarity coefficients calculated from protein profiles also revealed that isolates of different species of *Phytophthora*

Table 2. Similarity matrix based on similarity coefficients determined from electrophoretic patterns of native protein of three species of *Phytophthora*.

	P. capsici					*P. citrophthora*				
	I-CP/23	I-CP/25	I-CP/27	I-CP/76	I-CP/89	I-CP/26	I-CP/75	I-CP/92	I-CP/93	I-CP/96
P. palmivora										
(I-CP/122)	25	25	25	25	25	54·1	54·1	54·1	54·1	54·1
P. capsici										
I-CP/23		100	100	100	100	30·8	41·2	30·8	30·8	30·8
I-CP/25			100	100	100	30·8	41·2	30·8	30·8	30·8
I-CP/27				100	100	30·8	41·2	30·8	30·8	30·8
I-CP/76					100	30·8	41·2	30·8	30·8	30·8
I-CP/89						30·8	41·2	30·8	30·8	30·8
P. citrophthora										
I-CP/26							64·5	100	100	100
I-CP/75								64·5	64·5	64·5
I-CP/92									100	100
I-CP/93										100

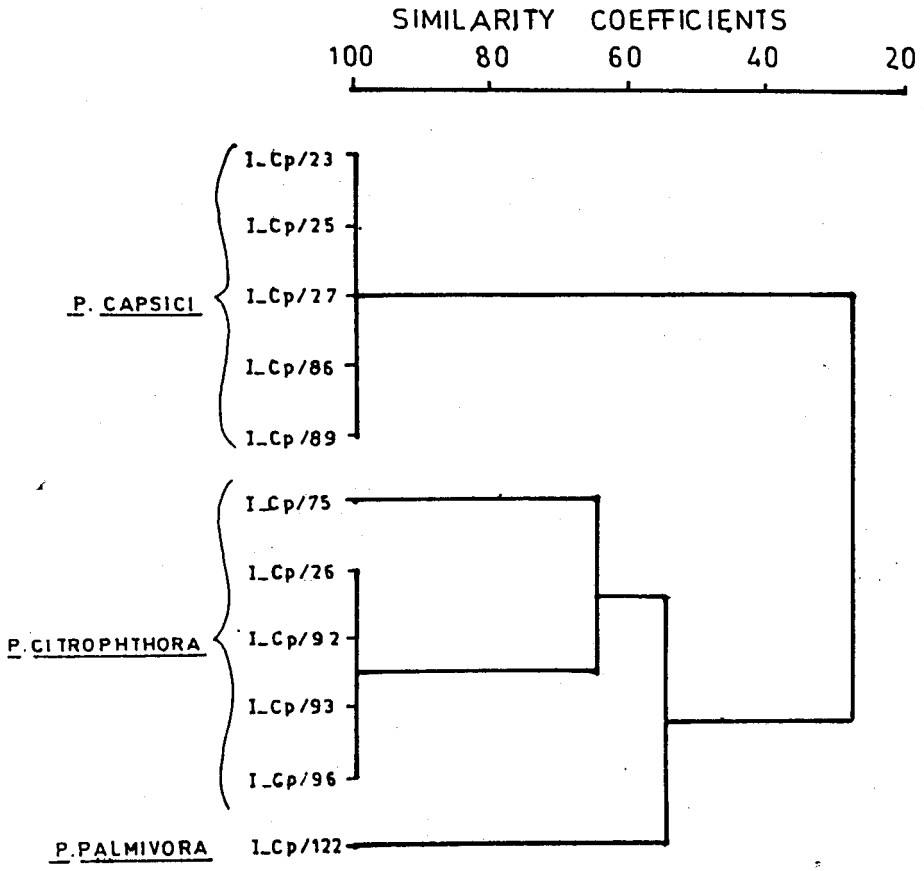

Figure 2. UPGMA cluster analysis of *Phytophthora* spp. based on similarity coefficients (SC) calculated from the electrophoretic patterns of native proteins.

formed different clusters. These results, thus, helped to confirm earlier identification of three species as *P. palmivora*, *P. capsici* and *P. citrophthora* on the basis of the cultural and morphological characters (Chowdappa and Chandramohanan 1993; Chowdappa *et al* 1993; P Chowdappa and R Chandramohanan, unpublished). Comparative Studies on *Phytophthora* isolates of cocoa from Africa and America revealed that *P. palmivora*, *P. megakarya* and *P. capsici* could be resolved into three distinct groups based on protein patterns and this variation has been correlated with differences in sporangial stalk length and other morphological characteristics (Kaosiri and Zentmyer 1980). Electrophoresis of native proteins was also found useful in separating and grouping of isolates of six species of *Phytophthora* encountered on deciduous fruit crops which could also be distinguished by cultural and morphological characters and cardinal temperature (Bielenin *et al* 1988). From the foregoing, it could be inferred that morphological and physiological differences between species of *Phytophthora* are reflected in differences in protein banding patterns.

In the present study, five isolates of *P. citrophthora* exhibited two recognizable types of banding patterns and therefore, they were clustered into two distinct sub groups. Similarly, de vallavieille and Erselius (1984) separated 77 isolates of *P. citrophthora* from citrus into two distinct groups (group A and group B) based on protein banding patterns. Studies on nuclear DNA polymorphisms by Goodwin *et al* (1990) have revealed two apparently unrelated groups in *P. citrophthora*, one from Brazilian cocoa and another from citrus, walnut, kiwi and cherry in California. These results were supported by mitochondrial DNA polymorphisms and isozyme studies (Förster *et al* 1990) which showed 40% genetic similarity between *P. citrophthora* of cocoa and *P. citrophthora* isolates of citrus-walnut group. Förster *et al* (1990) suggested separate founder effects from an ancestral population or convergent evolution from unrelated species as explanations for the elctrophoretic groups. A comparison of results from these studies support the concept that two groups of *P. citrophthora* from cocoa in India are probably genetically distinct. Thus, the results obtained with electrophoresis support the use of this approach as one of the aids in distinguishing both interspecific and intraspecific variation in *Phytophthora* spp. encountered on cocoa.

References

Bielenin A, Jeffers S N, Wilcox W F and Jones A L 1988 Separation by protein electrophoresis of six species of *Phytophthora* associated with deciduous fruit crops; *Phytopathology* **78** 1402–1408

Brasier C M 1983 Problems and prospects in *Phytophthora* research; in *Phytophthora*: *Its biology, taxonomy, ecology,* and *pathology* (eds) D C Erwin, S Bartnicki-Garcia and P H Tsao (St. Paul: American Phytopathological Society) pp 351–364

Brasier C M and Griffin M J 1979 Taxonomy of *Phytophthora palmivora* on cocoa; *Trans. Br. Mycol. Soc.* **72** 111–143

Chowdappa P, Chandramohanan R and Ramanujam P 1993 Occurrence of *Phytophthora capsici* on cocoa in Kerala; *Indian Phytopathol.* **46** 92–93

Chowdappa P and Chandramohanan R 1993 Morphological variability among isolates of *Phytophthora palmivora* causing black pod disease of cocoa in India; *J. Plantn. Crops* (Suppl.) **21** 129–133

de vallavieille C and Erselius L J 1984 variation in protein profiles of *Phytophthora*: Survey of a composite population of three species of citrus: *Trans. Br. Mycol. Soc.* **83** 473–479

Erselius L J and de vallavieille C 1984 Variation in protein profiles of *Phytopthora*; comparison of six species; *Trans. Br. Mycol. Soc.* **83** 463–472

Förster H, Oudemans P and Coffey M D 1990 Mitochondrial and nuclear DNA diversity within six species of *Phytophthora*; *Exp. Mycol.* **14** 18–31

Gallegly M E 1983 New criteria for classifying *Phytophthora* and critique of existing approaches; in *Phytophthora: Its biology, Taxonomy, ecology* and *pathology* (eds) D C Erwin, S Bartnicki-Garcia and P H Tsao (St. Paul: American Phytopathological Society) pp 167–172

Gill H S and Zentmyer G A 1978 Identification of *Phytophthora* species by disc electrophoresis; *Phytopathology* **68** 163–167

Goodwin P H, Kirkpatrick B C and Duniway J M 1990 Identification of *Phytophthora citrophthora* with cloned DNA probes; *Appl. Environ. Microbiol.* **56** 669–674

Guncu M and Cinar A 1989 Effect of culture conditions on the electrophoretic protein and acetylesterase patterns of some *Phytophthora* isolates; *Doga Turk Tarum ve ormancilik Dergisi* **13** 552–558

Hamm P B and Hansen E M 1983 *Phytophthora pseudotsugae*, a new species causing root rot of Dought-fir; *Can. J. Bot.* **61** 2626–2631

Hansen E M, Brasier C M, Shaw D S and Hamm P B 1986 The taxonomic structure of *Phytophthora megasperma*: Evidence for emerging biological species groups; *Trans. Br. Mycol. Soc.* **87** 557–573

Kaosiri T and Zentmyer G A 1980 Protein, esterase, and peroxidase patterns in the *Phytophthora palmivora* complex from cacao; *Mycologia* **72** 988–1000

Laemmli U K 1970 Changes of structural proteins during the assembly of the head of bacteriophage T4; *Nature (London)* **227** 680–685

Lowry O H, Roseborough N J, Farr A L and Randall R J 1951 Protein measurement with folin phenol reagent; *J. Biol. Chem.* **193** 265–275

Oudemans P and Coffey M D 1991 A revised systematics of twelve papillate *Phytophthora* species based on isozyme analysis; *Mycol. Res.* **95** 1025–1046

Sneath P H A and Sokal R R 1973 *Numerical Taxonomy: The Principles and practice of numerical classification* (Sanfrancisco; W H Freeman)

Stamps D J, Waterhouse G M, Newhook F J and Hall G S 1990 *Revised tabular key to the species of Phytophthora. Mycological papers* no. 162 (Kew; CAB International Mycological Institute)

Wilcox W F, Scott P H, Hamm P B. Kennedy J M, Brasier C M and Hansen E M 1993 Identity of *Phytophthora* species attacking raspberry in Europe and North America; *Mycol. Res.* **92** 817–837

Xu D Y, Huang H and Wang C D 1982 Polyacrylamide gel disc electrophoresis of proteins from species of *Phytophthora*; *Acta Mycol. Sinica* **1** 40–47

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