Identification of Phytophthora Species by Disc Electrophoresis

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ABSTRACT

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Soluble proteins from the mycelia of 30 isolates of *Phytophthora cinnamomi*, collected from 17 different hosts and from widely separated geographic locations, and of five isolates of *P. cactorum*, when fractionated by disc electrophoresis, yielded 22 and 26 bands with different densities. The two species differed markedly and each exhibited its distinct, characteristic protein pattern enabling us to identify them. With one exception, there was little or no variation in the protein patterns within the isolates of *P. cinnamomi*. Also, identical or nearly identical protein patterns of each species were obtained regardless of date of isolation, host, or geographic locality. No differences in protein patterns were seen between the mating types of *P. cinnamomi*.

Despite much research on *Phytophthora* spp. (12, 20, 46), the identification of most of the species is still difficult, time-consuming, and often uncertain or confusing. As in other groups of fungi, classification in the genus *Phytophthora* is predominantly based upon morphological and cultural characteristics (23, 43). Lack of, or difficulty of inducing asexual and sexual organs, which are essential for identification, is another limiting factor. Morphological variability of these structures, produced under the influence of different environmental and nutritional conditions, compounds the problem (9, 48).

Some progress has been made toward alleviation of some of these problems, however. In the last decade, several workers explored the usefulness of protein patterns by gel electrophoresis as an aid to taxonomy of a diverse group of microorganisms (2, 3, 10, 17, 18, 21, 22, 27, 29, 32, 34, 35, 37, 41, 42). Though this technique has not proved useful in several investigations (28, 36, 38, 40, 41), it was helpful in differentiating some pythiaceous fungi. Clare (4) differentiated species of *Pythium* by electrophoretic protein patterns in starch gel. Clare and Zentmyer (6) reported differentiation of *Phytophthora cinnamomi, P. citrophthora,* and *P. palmivora* by starch gel-electrophoresis. Gill and Powell (13) delimited *P. cactorum, P. fragariae,* and *P. sojae* by polyacrylamide gel electrophoresis and further demonstrated the usefulness of this technique for diagnostic purposes at the species rather than at the race level (14). Hall et al. (19), found little intraspecific variation in *Phytophthora* with the exception of *P. palmivora* which yielded two quantitatively different protein profiles; interspecific protein patterns, however, differed significantly.

Since there is considerable controversy on the utility of protein patterns for taxonomic purposes in fungi, we reinvestigated this area to ascertain whether the disc electrophoresis protein patterns of a large number of isolates of *P. cinnamomi* and of *P. cactorum* obtained from diverse geographic areas and hosts were sufficiently stable characteristics of the species for diagnostic purposes, and also to compare electrophoretically the A^1 and A^2 mating types of *P. cinnamomi*.

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MATERIALS AND METHODS

Isolates and culture media.—Thirty isolates representing A^1 and A^2 mating types of *P. cinnamomi* from 17 host plants and from widely separated geographic areas, and five isolates of *P. cactorum* (Table 1) were grown in a basal liquid glucose-yeast-peptone (GYP) medium of the following composition: D-glucose, 15 g; L-asparagine, 2 g; $FeSO_4 \cdot 7H_2O$, 1 mg; $CaCl_2 \cdot 2H_2O$, 10 mg; $MgSO_4 \cdot 7H_2O$, 0.1 g; KH_2PO_4, 0.47 g; K_2HPO_4, 0.26 g; Difco Bacto yeast extract, 1 g; Difco Bacto peptone, 7 g; thiamine hydrochloride 1 mg; 1 ml of minor elements in solution to give, in the final solution, 1 μg/ml Zn ($ZnSO_4 \cdot 7H_2O$), and 0.02 μg/ml of Cu ($CuSO_4 \cdot 5H_2O$), and Mo ($Na_2MoO_4 \cdot 2H_2O$), in 1 liter of demineralized water. Three small disks, each 5 mm in diameter, of mycelium and agar from the edges of an actively growing culture on cornmeal agar were added to 50 ml of GYP medium in each 250-ml Erlenmyer flask. Cultures were incubated at 25 C for 7 days. The contents of two flasks of each isolate were combined in a small, sterile blender cup (Eberbach 8580, 360-ml capacity) and blended at high speed for 5 to 10 sec. The homogenized mycelial suspension, 5 ml, was added to 100 ml of GYP medium in each 800-ml Roux bottle. Cultures were incubated at 25 C for 7 days. The mycelium was harvested by filtration onto Whatman No. 1 filter paper on a Büchner funnel followed by three washings with distilled water. The buffer-soluble proteins were extracted by grinding blotted dry mycelium with a pestle in a mortar containing acid-washed sand and phosphate buffer at pH 7.0 (0.1 M potassium monobasic phosphate and 0.1 M sodium dibasic phosphate). The mixture was centrifuged at 27,000 *g* (Sorvall Superspeed RC-2) for 1 hr. The resultant clear supernatant liquid

from the fungal extract was decanted and immediately used for electrophoresis. All glassware and equipment were prechilled and all operations were carried out at 4 C.

A disc electrophoresis (7, 26, 30) apparatus [Model 12 (CANALCO Corporation, Bethesda, MD 20014)] was used. Gel columns were prepared by filling glass tubes (70 mm × 5 mm internal diameter) first with 1 ml of 7% separating gel followed by 0.2 ml of spacer gel. A sample gel (0.2 ml) containing 350 μg of fungal protein as determined by the Lowry method (24) was pipetted over the spacer gel. Electrophoresis was carried out at room temperature (24-26 C), using a tris-glycine buffer at pH 8.2-8.5. A current of 5 ma per tube was applied until the tracking dye, bromophenol blue, had moved about 50 mm into the separating gel. The gels then were removed from the tubes, stained with amido black or Coomassie blue for 1 hr and destained with several changes of 7% acetic acid.

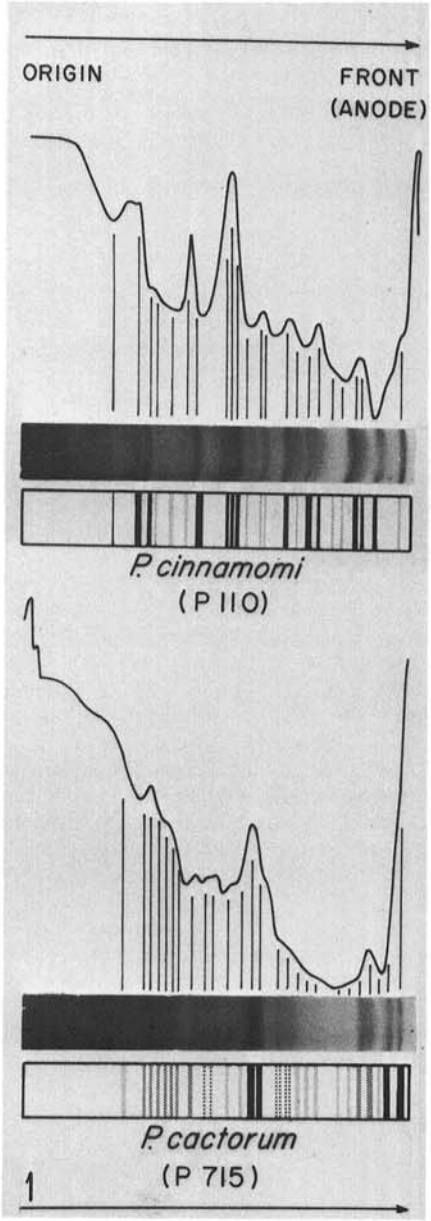

Fig. 1. Densitometer scans and corresponding electrophoretic patterns in polyacrylamide gel together with diagrammatic representation of protein bands characteristic of *Phytophthora cinnamomi* (P 110) and *P. cactorum* (P 715).

Protein patterns were evaluated on the basis of number, position, density, and width of the bands. The E_f values were calculated by expressing the mobility of each band in relation to the tracking dye. Destained gels were viewed and photographed on high-contrast copy film using diffuse white transmitted light. They were scanned with a Transtab Type D8 MK2 microdensitometer (Joyce Loebl and Co., Ltd., England). A diagrammatic presentation is given for isolates or species comparison to show the very faint or light bands in the gel easily visible to the naked eye but not distinct on the photograph or on the scan.

Six replications were observed in each run of each isolate and several runs were made from each protein preparation. The experiments were repeated with fresh extracts prepared at different times.

RESULTS AND DISCUSSION

The protein patterns of buffer-soluble proteins extracted from mycelium of each isolate of either species of *Phytophthora* examined were reproducible in different electrophoretic runs. Such profiles also were identical to that of a different culture of the same isolate grown at a different time under identical conditions. This information substantiated earlier investigations conducted on species of *Phytophthora* (6, 13, 19), *Pythium* (4), and other organisms (3, 8, 16).

Like earlier electrophoretic studies on species of *Phytophthora* (6, 13, 19) and other fungi (3, 4, 27), a distinct and characteristic protein pattern was obtained for each species studied (Fig. 1 and 2). Each of the 30 isolates of *P. cinnamomi* and the five isolates of *P. cactorum* resolved into 22 and 26 bands with different E_f values and densities. The protein patterns of all isolates of *P. cinnamomi,* with the exception of P 62, which appeared slightly different from the rest, where identical. Five isolates of *P. cactorum* were similar to each other, but distinct from isolates of *P. cinnamomi* in protein patterns. Similar results also were obtained by Chang et al. (3) who worked on *Neurospora crassa, N. intermedia, N. sitophila,* and a mutant strain of *N. crassa.*

Isolate P 62 is an unusual culture of *P. cinnamomi,* differing from most other isolates in its cultural morphology, and in its substantial growth at 33 C. Thus, it might be expected to differ somewhat electrophoretically.

Shechter et al. (35) and Shechter (33) reported quantitative differences between stock and fresh isolates and pigmented and nonpigmented isolates of species of dermatophytic fungi. Whitney et al. (45) found variation in protein patterns within two isolates, of different dates of isolation, of *Verticillium albo-atrum.*

Snider and Kramer (39, 40) found intraspecific as well as interspecific variation in the protein patterns of *Taphrina* spp. They believed that the genotypic change in these fungi maintained on agar media over a period of years since their isolation, was responsible for the variability in protein patterns. They recommended the

use of freshly isolated cultures or lyophilized cultures of fresh isolates for electrophoretic studies. This may be possible with certain groups of organisms (33, 35, 39, 40, 45). However, we did not detect any qualitative differences in protein patterns obtained from cultures of different dates of isolation (Table 1) of either *P.*

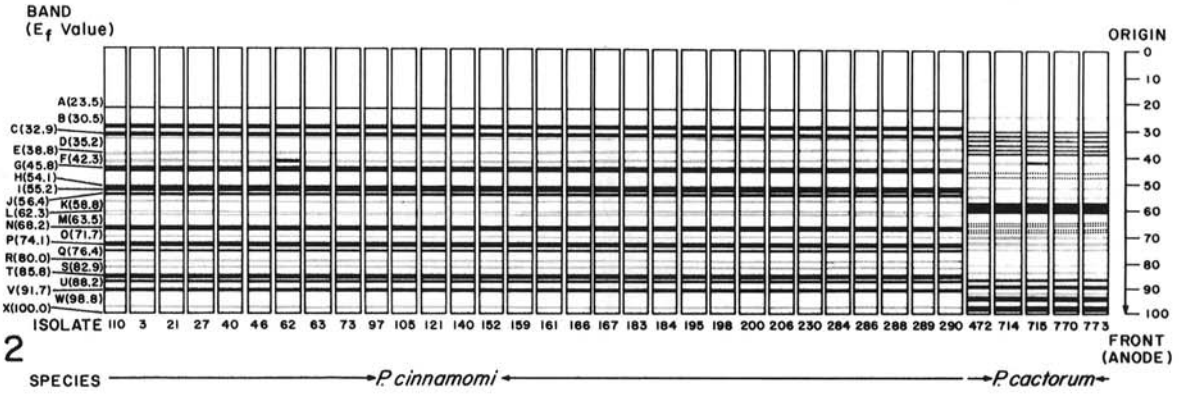

Fig. 2. Diagrammatic presentation of the electrophoretic patterns of protein of thirty isolates of *Phytophthora cinnamomi* and of five isolates of *P. cactorum*.

TABLE 1. Identification and origin of isolates of *Phytophthora cinnamomi* and *P. cactorum* which were compared by disc electrophoresis of mycelium extracts

Phytophthora species	Isolate no.	Mating type	Source host	Geographic origin	Year of isolation
P. cinnamomi	3	A^2	*Leucopogon verticillata*	West Australia	1965
	21	A^1	*Camellia* sp.	California (USA)	1966
	27	A^2	*Hibbertia cunninghamii*	New Zealand	1966
	40	A^2	*Persea americana*	California (USA)	1950
	46	A^1	*Macadamia ternifolia*	Hawaii (USA)	1961
	62	A^1	*M. integrifolia*	Hawaii (USA)	1961
	63	A^1	*Camellia japonica*	North Carolina (USA)	1962
	73	A^2	*Eucalyptus marginata*	West Australia	1964
	97	A^1	*Camellia* sp.	California (USA)	1968
	105	A^2	*Erica* sp.	England	...[a]
	110	A^2	*Cinnamomum burmanni*	Sumatra	1922
	121	A^1	*Persea americana*	Madagascar	1966
	140	A^2	*Prunus armeniaca*	Maryland (USA)	1970
	152	A^1	*Tristania conferta*	Australia	1971
	159	A^1	*Vitis vinifera*	South Africa	1971
	161	A^2	*V. vinifera*	South Africa	1971
	166	A^2	*Persea americana*	Costa Rica	1972
	167	A^2	*Persea americana*	Cameroons	1972
	183	A^1	Soil (*Nothofagus* sp.)	New Guinea	1972
	184	A^1	*Eucalyptus globoidea*	Australia	1972
	195	A^2	*Persea americana*	Argentina	1973
	198	A^2	*Persea americana*	Mexico	1973
	200	A^2	*Castanea sativa*	U.S.S.R.	...
	206	A^2	*Metrosideros collina* subsp. *polymorpha*	Hawaii (USA)	1973
	230	A^2	*Persea americana*	Mexico	1973
	284	A^2	*Erica* sp.	Switzerland	1970
	286	A^2	Soil (*Persea americana*)	California (USA)	1975
	288	A^2	*Pinus radiata*	California (USA)	1975
	289	A^2	*Cedrus deodara*	California (USA)	1975
	290	A^2	*Juniper* sp.	California (USA)	1975
P. cactorum	472		*Pyrus communis*	California (USA)	...
	714		*Syringa vulgaris*	...	...
	715		...	Great Britain	1921
	770		*Malus sylvestris*	Missouri (USA)	...
	773		*Malus sylvestris*	Poland	...

[a]Three dots indicate that the information is unknown.

cinnamomi or of *P. cactorum,* when compared with the type cultures, P 110 originally isolated by Rand in 1922 and P 715 used by Blackwell in 1921 (Table 1), respectively. Furthermore, identical or nearly identical protein patterns of isolates of each species were obtained regardless of the host or geographic locality from which the fungi were collected. This confirmed the earlier electrophoretic investigations on *Phytophthora* spp. (6, 13, 19) and *Pythium* spp. (4).

Although general protein patterns were found helpful in species differentiation, they did not aid in distinguishing mating types of *P. cinnamomi* (Fig. 2) [as was previously shown for physiologic races of *P. fragariae* (14)] and of *Puccinia coronata* var. *avenae* (36). In contrast, such patterns enabled Macko et al. (25) to distinguish two races of *Puccinia graminis* var. *tritici.* It appears that patterns obtained by gel electrophoresis may not be useful in characterizing subspecific taxa in some fungi.

The disagreement among some investigators concerning the usefulness of gel electrophoresis for taxonomic differentiation could be attributed to the nature of a taxon, suspected aggregate species (5, 11, 15, 18, 28, 31, 45), and the experimental conditions under which it is studied (1, 3, 5, 37, 45). The information presented in this paper and earlier (6, 13, 19) demonstrates that application of this technique under strictly standardized or uniform experimental conditions yields a unique characteristic protein pattern for a species. Such patterns may be integrated (as an additional taxonomic feature) with other conventional criteria (44) and used for precise identification of species of the genus *Phytophthora* (47).

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