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VIRULENCE AND ISOZYME VARIATIONS WITHIN FUNGI CAUSING Ascochyta BLIGHT OF Phaseolus vulgaris

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Fungal isolates obtained from foliar samples taken from bean with symptoms of *Ascochyta* blight in various regions of East Africa and Colombia were all related to *P. exigua* var. *diversispora* based on the symptoms produced on inoculated bean and isozyme analysis. Isozyme banding patterns revealed genetic variations between African and American strains of *P. exigua* var. *diversispora* and within the African isolates of this fungus.

INTRODUCTION

The literature reports several species of fungi which are considered to be responsible for *Ascochyta* blight. *Phoma exigua* Desm. var. *diversispora* (Bub.) Boerema causes severe damage in bean (*Phaseolus vulgaris L.*) and cowpea (*Vigna unguiculata* (L.) Walp.) in many areas of Latin America, the United States, Europe, and Africa (Boerema *et al.*, 1981); leaves infected with this fungus bear large necrotic spots, greyish brown in color, often with concentric rings of pycnidia. *Phoma exigua* Desm. var. *exigua*, formerly known as *Ascochyta phaseolorum* Sacc., is another agent of *Ascochyta* blight; spore characteristics of this organism are similar to those of *P. exigua* var. *diversispora*, but symptoms are different, consisting of small specks and spots on older leaves and pods. A third agent causing *Ascochyta* blight, *Stagonosporopsis hortensis* (Sacc. & Malbr.) Petr., formerly known as *Ascochyta boltshauseri* Sacc., is readily distinguished from both var. of *Phoma exigua* by the occurrence of large, frequently multi-septated conidia (Boerema *et al.*, 1981). *S. hortensis* induces small reddish brown spots on leaves, pods, and stems.

Traditionally, the classification of the fungi causing *Ascochyta* blight was based primarily on morphological characters, leading to taxonomic confusion mainly between *P. exigua* var. *diversispora* and *P. exigua* var. *exigua*, thus hampering the rational selection of resistant bean genotypes; therofore, other methods of identification are needed.

The possible taxonomic interest of the technique of gel electrophoresis to solve problems of genetic variability has been recognized (Burdon and Marshall, 1983). Enzyme banding patterns were used to study the variation within formae speciales and between species of a wide range of fungi (Burdon and Marshall, 1981). At the physiological race level, however, few differences were detected (Burdon *et al.*, 1981). The purpose of our study was to characterize the pathogenicity of various fungal isolates of different origins obtained from foliar material showing symptoms of *Ascochyta* blight, and to study the correlative variations of the electrophoretic banding pattern of selected enzymes within these isolates.

MATERIEL AND METHODS

Fungal material

Dr. W.M. Loerakker, "Plantenziektenkundige Dienst" (Wageningen, The Netherlands), provided reference strains PD 79/61 (=strain R1) of *P. exigua* var. *diversispora* originating from Kenya, and PD 79/269 (=strain R2) of *S. hortensis* originating from The Netherlands.

Dr. E.S. Van Reenen-Hoekstra, "Centraal Bureau voor Schimmelcultures" (Baarn, The Netherlands) gave us strain CBS 729.68 (=strain R3) of *P. exigua* var. *exigua*, originating from Brazil.

Dr. M. Gerlagh, "Instituut voor Plantenziektenkundig Onderzoek" (Wageningen, The Netherlands), donated strain 4.45 (=strain R4) of *P. exigua* var. *diversispora* originating from Rwanda, strain 50987 (=strain R5) of *P. exigua* var. *diversispora* originating from Guatemala, strain 40987 (=strain R6) of *P. exigua* var. *exigua* originating from Guatemala, and strain COL 5 (=strain R7) of *S. hortensis* originating from Colombia.

Eighteen isolates were obtained from leaves of *P. vulgaris* et *Vigna sp.*, showing severe symptoms of *Ascochyta* blight collected in different locations in Latin America and East Africa (table 1). Foliar lesions were treated 20-30 seconds in $AgNO_3$ and then incubated on oat meal agar (OMA) at 26°C, 16 h day/8 h night to allow the pathogen to grow out for isolation. Fungal strains were maintained on OMA medium.

Inoculation and incubation of the plants

Plantlets of *P. vulgaris* (cv. Ica Llano Grande obtained from CIAT, Colombia, and Karama Demi obtained from ISABU, Burundi) were inoculated at the first trifoliolate leaf stage by run-off spraying of leaves with an aqueous spore suspension (3×10^6 spores/ml containing 0,1% Tween 20) prepared from 10 day-old colonies on OMA.

Inoculated plants, arranged in a randomized complete block design (using three plants per fungal strain), were maintained for 3 hr under a relative humidity of about 70%, and were then transferred and maintained for 15 days in a cabinet at 20-22°C with a mist-type vaporizer operating continuously.

Disease assessment was made during 15 days following inoculation by daily evaluation of the percentage infected leaf area per plant.

Extraction and electrophoresis of enzymes

Isolates were grown on potato dextrose broth (PDB) in flask seeded each with two plugs excised from a fungal colony growing on OMA, and incubated 6 days in the dark at 24°C. The cultures then were filtered through mulsin and rinsed with distilled water. Five g of mycelium were ground in a motar, with addition of dry ice, in 5 ml O.2M Tris buffer pH 8.5 containing sucrose (1M) and mercaptoethanol (0.056M). Homogenization was obtained in a French press at 7 kg/cm^2. The crude extract was centrifuged at 30,000 g for 1 h and supernatant maintained at -20°C.

Disc electrophoresis on polyacrylamide gel was performed according to Wetter and Dyck (1983). The gels were assayed for a range of enzymes (table 2) by immersion in the reaction mixtures containing appropriate substance of the different enzymes (Vallejos, 1983).

Electrophoretic patterns were interpreted according to the methods of "the principal components analysis" (PCA) and "the hierarchical cluster analysis" (HCA) (Dagnelie, 1975).

Table 1: Fungal isolates from diseased leaves of *Phaseolus sp.* and *Vigna sp.* collected in South America of East Africa

Isolates	Host plant	Locations[1]	Altitude	Collector	Year
S1	*P. vulgaris*	Rutagama BDI	1600m	Perreaux-ISABU[2]	1985
S2	*P. vulgaris*	Mosso BDI	1250m	Perreaux-ISABU	1986
S3	*V. glabrescens*	Mosso BDI	1250m	Perreaux-ISABU	1986
S4	*V. radiata*	Mparambo BDI	900m	Perreaux-ISABU	1986
S5	*V. mungo*	Mosso BDI	1250m	Perreaux-ISABU	1986
S6	*V. unguiculata*	Bujumbura BDI	800m	Perreaux-ISABU	1986
S7	*V. unguiculata*	Mparambo BDI	900m	Perreaux-ISABU	1986
S8	*P. vulgaris*	Murangwe BDI	1470m	Perreaux-ISABU	1986
S9	*P. vulgaris*	Ijenda BDI	2300m	Perreaux-ISABU	1986
S10	*P. vulgaris*	Iheta BDI	1600m	Perreaux-ISABU	1986
S11	*P. vulgaris*	Rutegama BDI	1700m	Perreaux-ISABU	1986
S12	*P. vulgaris*	Popayan COl	1700m	Baudoin-FSAGx[3]	1985
S13	*P. vulgaris*	Sydundoy COl	2000m	Baudoin-FSAGx	1985
S14	*P. lunatus*	Rio Negro COL	2600m	Schmit-CIAT[4]	1986
S15	*P. vulgaris*	Popayan COL	1700m	Baudoin-FSAGx	1987
S16	*P. vulgaris*	Quito EQU	2700m	Baudoin-FSAGx	1987
S17	*P. vulgaris*	Bukavu Zar	1635m	Perreaux-ISABU	1986
S18	*P. vulgaris*	Mulungu Zar	1700m	Perreaux-ISABU	1986

[1] BDI = Burundi, COL = Colombia, EQU = Equator, ZAR = Zaïre

[2] ISABU = Institut des Sciences Agronomiques du Burundi, Burundi

[3] FSAGx = Faculté des Sciences agronomiques de Gembloux, Belgium

[4] CIAT = Centro Internacional de Agricultura Tropical, Colombia

Table 2: Enzymes studied after protein electrophoresis

Enzyme	Enzyme Commission Number	Abbreviation
Acid phosphatase	3.1.3.2.	ACP
Alcohol dehydrogenase	1.1.1.1.	ADH
Alkaline phosphatase	3.1.3.1.	AKP
Catalase	1.11.1.6.	CAT
Esterase	3.1.1.1.	EST
Galactose dehydrogenase		GaDH
Glutamate dehydrogenase	1.4.1.3.	GDH
Malate dehydrogenase	1.1.1.37.	MDH
Peroxydase	1.11.1.7.	PER
Shikimic dehydrogenase	1.1.1.25.	SDH

RESULTS

Symptoms induced by the fungal strains

Symptoms of *P. exigua* var. *diversispora* (reference strains R1, R4, or R5) on *P. vulgaris* (Karama demi or Ica Llano Grande), appeared 2-3 days following inoculation. Rings of pycnidia formed on the surface of the necrotic spots, and leaves collapsed when the necrotic lesions reached about half their surface (figure 1). Also, necrotic specks formed along stems and on nodes, which turned black approximately 10 days after inoculation.

After inoculation with *S. hortensis* (reference strain R7 or R2), or *P. exigua* var. *exigua* (reference strains R3 or R6), the percentage of infected leaf remained less then 10% with no symptoms on stems or nodes.

Symptoms induced by isolates S1 to S18 originating from South America or East Africa, all looked similar to those of the reference strains (R1, R4, or R5) of *P. exigua* var. *diversispora*.

Enzymes analysis

No activity for catalase, malate dehydrogenase, peroxydase, or shikimic dehydrogenase were detected for the isolates of fungi tested. Acid phosphatase and alkaline phosphatase were invariant for all fungal isolates examined. The banding patterns for alcohol dehydrogenase, esterase, galactose dehydrogenase, glutamate dehydrogenase, varied within the eighteen fungal isolates and the seven reference strains (table 3).

Table 3. Enzymatic bands observed for 6 enzymes and 25 fungal isolates.

		Fungal isolates																								
Enzyme	Rf	R1	R2	R3	R4	R5	R6	R7	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18
Est A	39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-
Est B	43	0	-	-	0	0	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Est C	46	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Est D	50	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Est E	67	-	-	-	-	-	-	-	0	-	0	0	-	-	-	-	-	0	-	-	-	-	-	-	0	0
Est F	75	-	-	-	-	-	-	-	0	-	0	0	-	-	-	-	-	0	-	-	-	-	-	-	0	0
Est G	76	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Est H	84	0	-	0	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Est I	86	-	-	-	-	0	-	-	-	-	0	-	-	-	-	0	-	0	-	0	0	0	0	-	0	0
Est J	87	-	0	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Est K	88	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Est L	90	-	-	-	-	-	-	-	-	0	-	-	-	0	0	-	0	-	0	-	-	-	-	-	-	-
Est M	92	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	0	0	0	-	-
Est N	95	-	0	-	0	-	-	-	0	0	0	0	0	0	0	0	0	0	0	-	-	-	-	0	0	-
Alc A	52	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	-	-
Alc B	54	-	-	0	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alc C	56	-	-	-	-	-	-	-	-	0	0	-	-	-	-	0	0	-	0	-	-	-	-	-	0	0
Alc D	58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-
Alc E	61	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	-	0	0
Alc F	63	-	0	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gal A	45	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gal B	55	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-
Gal C	56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	-	-	-	-
Gal D	57	-	0	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gal E	61	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0	-	-	-
Gal F	63	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	0	-	-	-	-	-	-	-	0	-
Gal G	74	0	-	-	-	-	-	-	0	-	0	0	0	0	-	0	0	0	0	-	-	-	-	-	0	0
Glu A	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0	0	-	-
Glu B	52	-	0	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glu C	56	0	-	-	-	-	-	-	0	0	0	-	-	-	-	0	0	0	0	-	-	-	-	-	0	0
Glu D	60	-	-	-	-	-	-	-	-	0	0	-	-	-	-	0	0	-	-	-	-	-	-	-	0	-
P. Ac	77	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. Alc	74	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 : presence of the corresponding band

- : absence of the corresponding band

Est : esterase
Alc : alcohol dehydrogenase
Gal : galactose dehydrogenase

Glu : glutamate dehydrogenase
P. Ac : phosphatase acid
P. Alc : phosphatase alkaline

Principal component analysis (PCA) showed that the 31 enzymatic bands, reflecting the enzymatic polymorphism of the 25 fungal isolates or strains, could be grouped into eight main components, responsible for 89% of the total variability. The first three axis of PCA explained 57% of the total variability (24.2% for axis Z1, 18.5% for axis Z2 and 14.6% for axis Z3). Figures 2 and 3 indicate the position of the 25 fungal isolates or strains in plans Z1-Z2 and Z1-Z3, and their association into six different groups.

No fungal isolates originating from South America or East Africa were associated with groups G1 or G2, corresponding to reference strains of *S. hortensis* (R2 and R7) or reference strains of *P. exigua* var. *exigua* (R3 and R6).

All South-American isolates (S12, S13, S14, S15 and S16) were associated into group G3, together with the reference strain R5 (*P. exigua* var. *diversispora*) originating from Guatemala. All African isolates (S1 to S11) belonged to groups G4a, G4b or G4c. Group G4c contained the reference strains R1 and R4 of *P. exigua* var. *diversispora* , originating from Africa.

Figure 4 represents the dendogram obtained after hierarchical cluster analysis (HCA), with seven groups explaining 76% of the variability of the enzymatic polymorphism. Both HCA and CPA led to a similar classification of the 25 fungal isolates or strains, except that group G4b, as defined with CPA, split into two groups (5 and 6) in the HCA method.

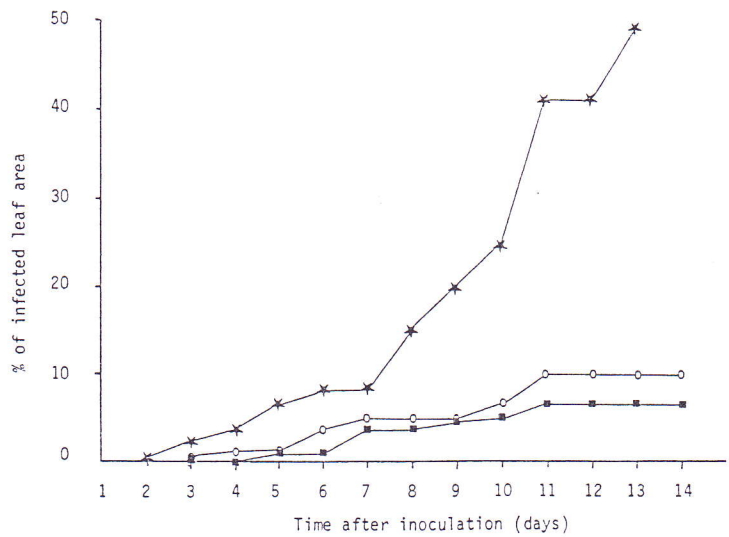

Figure 1. Evolution of the percentage of infected leaf area on *P. vulgaris* (Karama Demi) inoculated with *Phoma exigua* var. *diversispora* (R1: ★–★), *S. hortensis* (R2: o–o), *P. exigua* var. *exigua* (R3: ▪–▪).

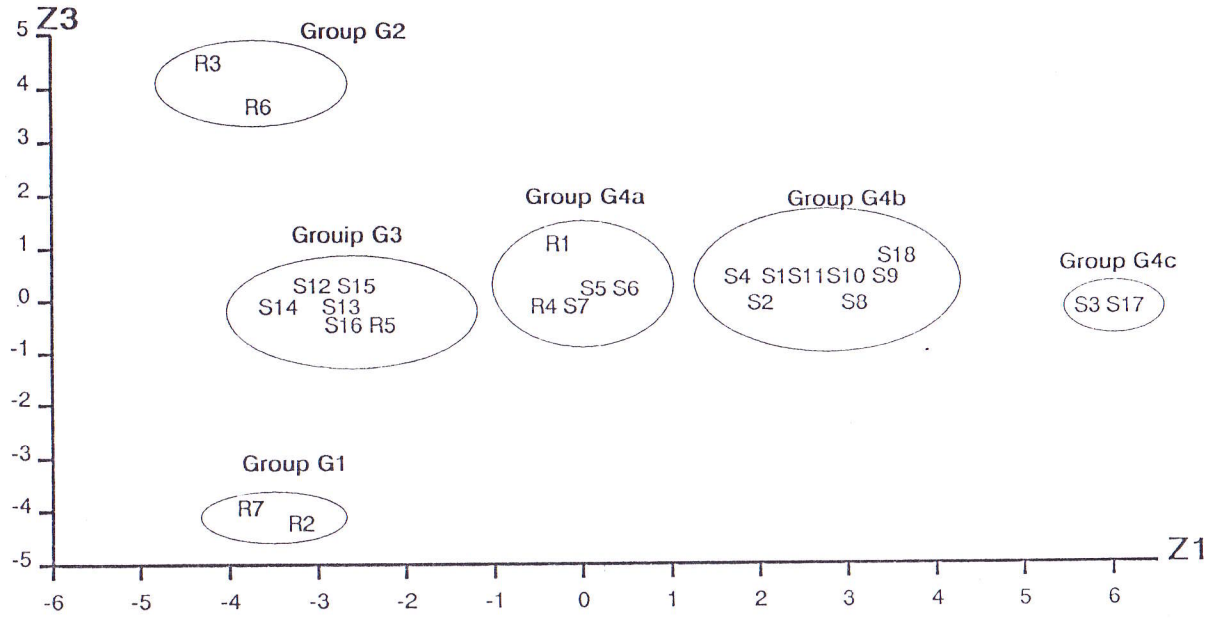

Figure 2: Position of the 18 isolates and 7 reference strains of *S.hortensis,P.exigua Var.exigua ;P.exigua var.diversispora* on the plan Z3-Z1,on the basis of the princial components analysis.

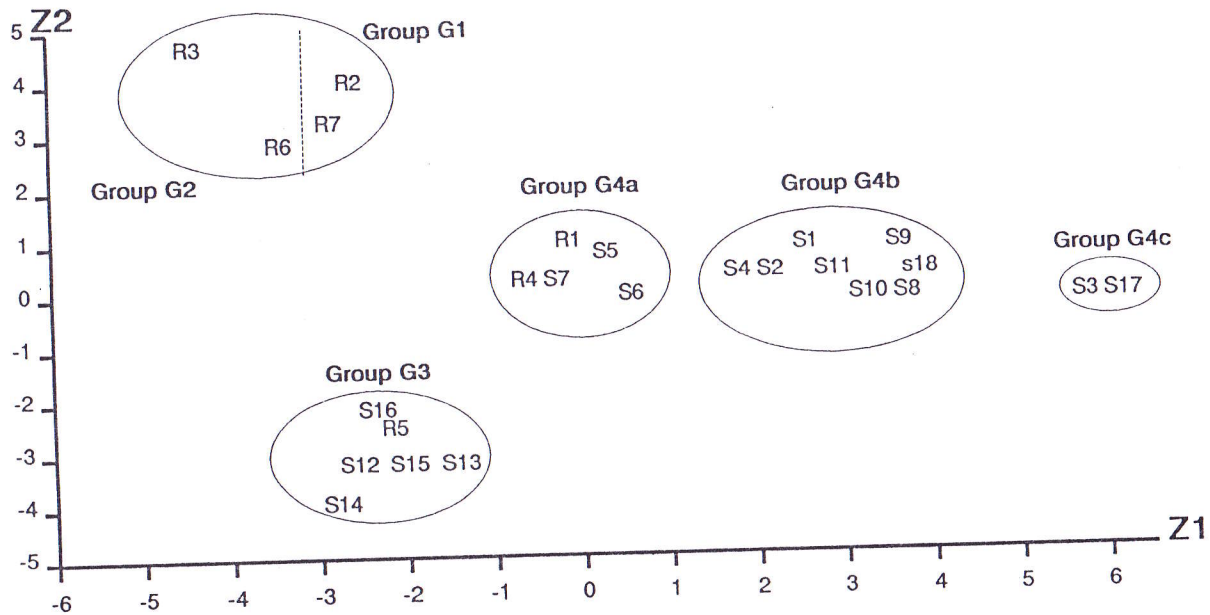

Figure 3: Position of the 18 isolates and 7 reference strains of *S.hortensis,P.exigua Var.exigua ;P.exigua var.diversispora* on the plan Z2-Z1,on the basis of the princial components analysis.

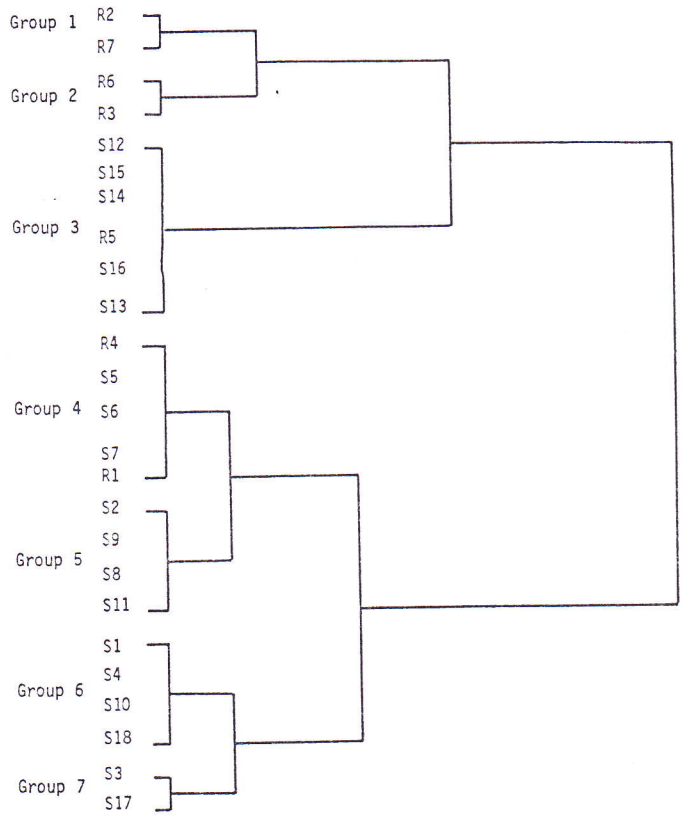

Figure 4. Dendogram resulting from hierarchical cluster analysis from the electrophoretic banding pattern of the isolates and reference strains of *Ascochyta* blight agents.

DISCUSSION

Fungal isolates obtained from foliar samples taken in the field from beans with symptoms of *Ascochyta* blight in various regions of Africa and South America were all related to the species *P. exigua* var. *diversispora* on the basis of symptoms produced on inoculated bean.

These results confirm that *P. exigua* var. *diversispora* is highly pathogenic on beans, whereas *P. exigua* var. *exigua* and *S. hortensis* are moderately pathogenic.

Isozyme analysis is a versatile and inexpensive technique to be used to reveal genetic variations among organisms, for the clarification of fungal taxa, and for the identification of fungal cultures to the species or subspecies level.

This technique has been used successfully to distinguish species of *Peronosclerospora* (Micales *et al.*, 1988), Endothia and *Cryphonectria* (Micales *et al.*, 1987).

In our study, the high degree of dissimilarities between reference strains of *S. hortensis*, *P. exigua* var. *exigua* and *P. exigua* var. *diversispora* agrees with their traditional separation into different entities according to classical taxonomic criteria. This point is particulary important to distinguich *P. exigua* var. *diversispora* from *P. exigua* var. *exigua*, the morphological characteristics of both species being very similar.

In accordance with this symptomatological study, the electrophoretic pattern of the isolates originating from East Africa or South America were linked to the corresponding reference strains of *P. exigua* var. *diversispora* .

The study of the population genetics of specific fungi is another area in which isozyme analysis can be used.

Difference in cultural characteristics between the isolates of *P. exigua* var. *diversispora* from Colombia, and those from Western Europe, has already been observed (Gerlach, personal communication). According to electrophoretic patterns, it is noteworthy that all isolates collected from South America were associated within a specific group, including the Americain reference strain of *P. exigua* var. *diversispora* . The analysis of the status of the African isolates seems more complex: these isolates (together with the reference strains originating from Africa) were associated into three different groups (according to PCA method) or four groups (according to HCA method), independently of host plant, altitude or area of origin.

Such intraspecific differences between isozymes from isolates in the Eastern hemisphere, or of the Americas, has been observed with the soybean rust fungi *Phakospora pachyrhizi* (Bonde *et al.*, 1988). The low frequency of shared putative alleles indicates that two populations of *P. pachyrhizi* are involved in causing rust on soybean: one in Asia and Australia, and another one in America (Brazil and Puerto Rico).

However, such intraspecific differences are rarely related to differences in pathogenicity. One exception was the correlation, in asexual populations of *Puccinia graminis f. sp. tritici* between isozyme phenotypes and nine major virulence groups within the populations of this fungus in the United States. Within each virulence grouping, all individual isolates had exactly the same isozyme banding patterns (Burdon and Roelfs, 1985b). These authors also used isozyme patterns of *Puccinia recondita* to trace independent introductions of this pathogen into the United States (Burdon and Roelfs, 1985a).

In the case of *Ascochyta* blight of bean, it is difficult to speculate on the significance of the differences in isozyme banding patterns between Africa and American strains of *P. exigua* var. *diversispora* and within the African isolates of this fungus. Nevertheless, such differences in isozyme banding patterns between *Ascochyta* blight agents could be connected to the fact that *Phaseolus* species show different levels of resistance to given African or American isolates of *P. exigua* var. *diversispora* (Obando *et al.*, 1988).

Nothing is known about the genetics of *P. exigua* var. *diversispora*, so that the analysis of the isozyme pattern cannot be related to phenotypical markers. The interpretation of our electrophoretic data is thus restricted to a simple "band counting" which provides us a static view of the genetic population structure of the pathogen. Isozyme analysis, when combined with genetic knowlegde of the pathogen, allows to

control plant pathogens.

For *Ascochyta* blight of *Phaseolus*, isozyme analysis appeared to be a powerful tool to measure the genetic variability within and between incipient fungal species. The technique proved useful to characterize fungal taxa and to identify genetic variation among *P. exigua* var. *diversispora*, which is of uppermost importance for the selection of resistant genotypes of *Phaseolus sp.*

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