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CHARACTERIZATION OF TWO BEAN PATHOGENS
BY ISOZYME ANALYSIS

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CHARACTERIZATION OF TWO BEAN PATHOGENS BY ISOZYME ANALYSIS

By

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ABSTRACT

CHARACTERIZATION OF TWO BEAN PATHOGENS BY ISOZYME ANALYSIS

By

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Isozymes of different pathogenicity groups of two bean fungal pathogens were analyzed. Isozyme patterns of Latin American, African, and North American isolates of Phaeoisariopsis griseola revealed polymorphism and monomorphism for catalase, esterase, and leucine aminopeptidase enzymes. Latin American isolates were polymorphic, while African isolates were monomorphic for these enzymes. Isozyme pattern 1 of esterase, catalase, and leucine aminopeptidase is suggested as the common ancestry for all isolates of P. griseola.

Culture conditions affected banding patterns in isozyme analyses of Colletotrichum lindemuthianum isolates. Samples grown 14 days on M1 medium exhibited the best electrophoretic characteristics.

Electrophoretic analysis of C.lindemuthianum isolates from Colombia and Europe revealed polymorphism for esterase, catalase, phosphoglucomutase and diaphorase but monomorphism for leucine aminopeptidase and glucosephosphate isomerase. Isozyme patterns were not related to physiological races nor to geographical origin of isolates, instead patterns reflected a complex genetic structure of the species.

To my mother with love

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INTRODUCTION

Beans (Phaseolus vulgaris L.) are one of the most important food crops in Latin America and in the highlands of eastern and southern Africa, where they are considered as one of the most important sources of protein and calories for the poor people(27).

High susceptibility of the crop to diseases is one of the main factors limiting improved productivity. A wide variety of pathogens and pests are responsible.

Research efforts in the bean crop have concentrated on the most economically important bean diseases, which include: anthracnose, angular leaf spot (ALS), rust and common bacterial blight (27). Angular leaf spot of beans caused by Phaeoisariopsis griseola (Sacc.) Ferr, has a wide distribution and is considered a major problem in many of the bean growing areas. Its occurrence is often sporadic but when environmental conditions are favorable, infection can reach epidemic levels. The disease is economically important in many bean growing areas of Latin America, particularly Brazil. In Africa, the disease is very widespread, having great importance in the Great Lakes area (Burundi, Rwanda, and Zaire) where it is endemic.

Previous investigations have indicated that pathogenic variability is common in P. griseola (22, 23, 30, 31, 55, 63). Reactions of many bean lines or varieties to the ALS

pathogen vary considerably from one location to another. Thus research has illustrated the pathogenic variation present in the ALS fungus in Latin America and Africa, and the need for identification of ALS resistance in bean germplasm (22, 27). Pathogenic and isozymic variation of the fungus populations of Latin American and African isolates of P. griseola (22, 23, 30, 31, 55, 63).

Anthracnose of beans is caused by Colletotrichum lindemuthianum (Sacc. and Magn.). The disease constitutes another of the most important factors contributing to serious yield reductions. The anthracnose pathogen exhibits extensive pathogenic variation which accounts for the different disease reactions of many bean varieties from one location to another. The fact that anthracnose is a seed borne disease, further adds to its importance as a yield reducing disease.

Physiological specialization or races in C. lindemuthianum was first observed in 1911 (7). Varieties resistant to repeated inoculation made with one isolate in one locality, became seriously infected when inoculated with an isolate from another locality. Barrus (7) reported two different forms or variants of the organism that were pathologically different from each other. These two variants of the pathogen were later designated as alpha and beta races. Later in 1922 a third race was discovered and designated as gamma (21). Since then many races have been discovered and designated by other Greek letters.

Recently molecular techniques have allowed the identification of phenotypic markers that permit a more comprehensive analysis of fungal pathogens. Isozyme markers have been extensively used with several plant and animal species and they are beginning to be employed more extensively to study variability within phytopathogenic fungi.

Electrophoretic analysis of proteins and enzymes has been used considerably in fungal taxonomy but less emphasis has been put on the characterization of strains, formae speciales or isolates of an organism with variable degrees of pathogenicity (2). There are at least three major areas in which isozyme analysis can be used. These include: the classification and delineation of fungal taxa, the identification of fungal cultures to the species or subspecies level, and the study of the genetics, including population genetics of specific fungi (47).

Considering all these factors, isozymes patterns could be used to evaluate the pathogenicity of phytopathogenic fungi by looking at the relationship between isozyme patterns and pathogenicity in different isolates of plant pathogens.

The main objectives of the present study were:

1. To confirm the results of previous isozyme studies with P. griseola (31) and to determine the best enzyme-buffer system combinations to detect variation in pathogen populations.
2. To study the correlation between isozymic variation in

P. griseola and geographic origin of isolates, pathogenicity and host gene pool characteristics.

3. To study the race composition of C. lindemuthianum isolates from Colombia and to determine its correlation with isozyme variation.

4. To determine the cultural factors affecting isozyme variation in C. lindemuthianum populations from Colombia and a group of European races of the fungus.

LITERATURE REVIEW

The reasons for studying the genetics of plant pathogens came from the need to better understand of pathogenicity.

Development and introduction into commercial production of resistant varieties has resulted in the evolution of plant pathogens into new variants that could overcome the resistance. The new physiologic races of plant pathogenic fungi could be identified by inoculation of isolates onto a group of differential varieties of the host (65).

Development of physiologic races of plant pathogens in populations of the host has been based on two main criteria: a) the gradual breakdown of resistance in a variety in a given area and, b) the large difference in varietal reactions at different locations or countries (25). There are two important factors in developing durable genetic resistance to the angular leaf spot (ALS) pathogen: the first will be defining variability in the pathogen; the second, is the knowledge of the ability of this fungus to "evolve" into new races. An understanding of these two factors should allow the development of bean cultivars with a broad spectrum of resistance to the ALS pathogen (1).

Previous studies reported pathogenic variability in P. griseola (3, 15, 22, 44, 55, 63). In 1983 Buruchara (22) grouped 21 isolates from Colombia and one isolate from

Wisconsin (USA) into seven pathotypes on the basis of their pathogenicity on six bean cultivars. He designated isolates as pathotypes instead of races, because genetic purity of the host differentials was not determined.

Correa (30) described five pathogenicity groups after inoculation of isolates of P. griseola to 12 bean differential cultivars with 30 isolates of P. griseola. Michigan and Wisconsin isolates of P. griseola were placed in a single group while African and Latin American isolates comprised the other four. Large seeded bean types were severely infected by the less pathogenic isolates.

Correa (31) subsequently determined additional pathogenic variability in P.griseola. Forty-two isolates of the pathogen from Latin American and African were separated into fourteen different pathogenicity groups on the basis of their reactions on eight differential bean cultivars. Pathogenic variation was observed for isolates both within and between different African and Latin American countries.

Physiological specialization or races of C. lindemuthianum were first observed by Barrus in 1911 (7), who reported alpha and beta races. In the following years several researchers reported new races of C.lindemuthianum and designated them with Greek letters. In other instances, researchers have used different codes to designate races of C.lindemuthianum such as Group I-III in Mexico, 1-8 in Australia, A-X in Germany and, races PV6, D10, I4, E8b and L5 in France (4, 11, 42, 49, 54, 62).

Guzman and Donado (38) studied Colombian isolates of C. lindemuthianum, and determined that beta was the most prevalent race in the Popayan area of Colombia. Later wider variability in C. lindemuthianum in Colombia was detected in the Nariño area, where beta and epsilon races, as well as a variant of the alpha race were detected (52).

In 1986 Cobo (29) reported that none of the known European races were present in group of seventeen Colombian isolates. The Colombian isolates were similar to Brazilian and Mexican races, where delta, Mexican II, alpha, Brazilian II and beta groups were predominant. Race B.A-10 belonging to the delta group was determined as the most prevalent of the Colombian isolates of C.lindemuthianum.

It has been hypothesized that the origin of physiological races of C.lindemuthianum could be due to mutation or parasexual recombination occurring between existing races (6, 21, 24, 33). Batista and Chavez in 1982 (8) reported that new physiological races of C.lindemuthianum resulted from sexual recombination, however this work has not been confirmed.

Physiological and epidemiological studies have provided evidence to suggest that physiological races are genetically uniform entities (65).

In order to clarify the definition of physiological race as a homogeneous population, it is important to evaluate the available data on the genetic composition of such races. First of all, isolates representative of the fungal

population must be evaluated on a set of differential cultivars and secondly, evaluation must be done on one or more independent traits such as colony morphology, rate of growth, and sporulation. Isozyme analysis provides a set of secondary characters with the advantage of being very relatable to the pathogen's genome (65).

Electrophoretic techniques for protein and enzyme analysis have been applied extensively in fungal taxonomy studies, but less emphasis has been put on their application to the differentiation and characterization of strains, formae speciales, or isolates of an organism with variable degrees of pathogenicity.

Recently isozyme analysis has been useful for the analysis and quantification of genetic variability in fungi such as Agaricus, Entomophthora, Neurospora, Peronosclerospora, Phytophthora and Puccinia. Clare et al. in 1968 (28) indicated that fungal taxonomy has largely been based on morphology, particularly of sexual reproductive structures. Thus the many fungi that lack a sexual stage are difficult to identify.

Electrophoretic patterns of soluble enzymes and other proteins are a direct manifestations of the cell's genetic makeup, and they could provide a valuable tool in taxonomy. Clare et al. (28) studied twenty species of fungi, including representatives of Phycomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes, and found that oxidoreductase isozyme patterns were of potential value in fungal taxonomy at the

subspecific level.

Stout and Shaw (60) determined that intraspecifically, enzyme patterns in mycelial extracts of twenty species of Mucor were virtually identical. Pattern differences resulted from the occurrence of different alleles in the population. Interspecifically, differences were marked. Huguenin et al. (39) differentiated Colletotrichum falcatum and C.graminicola on the basis of enzymatic polymorphism and pathogenicity: enzymes studied were leucine aminopeptidase, acid phosphatase and esterase.

Burdon and Marshall (16) determined isozyme variation between species and formae speciales of the genus Puccinia. Results clearly indicated that a great of differentiation between different species and formae speciales of the genus with respect to the electrophoretic banding patterns detected for eight different soluble enzymes. High degrees of dissimilarity between Puccinia species agreed with their separation according to traditional taxonomic criteria.

Backhouse et al. (5) determined that separation of Botrytis species by electrophoretic analysis agreed with the initial separation into species using traditional taxonomic criteria. Each species appeared distinct.

Electrophoretic patterns of sporangiospore proteins are used as taxonomic characters for various isolates of the genus Rhizopus (53).

Micales et al. (48) studied taxonomy within the genus Peronosclerospora and found that isolates of P.sorghi from

Thailand were not related to any other isolates. Isolates of P.sacchari and P.philippinensis exhibited identical phenotypes for 22 enzymes and probably represent a single species. P.maydis shared phenotypes with P.sacchari and P.sorgi isolates from Thailand.

Isolates of Glomerella cingulata obtained from different host plants were assayed qualitatively for soluble proteins, acid and alkaline phosphatase and esterase. Results indicated that all isolates were representative of a single species (59).

Electrophoretic studies of Australian, North American and European isolates of Sclerotinia sclerotiorum and related species resulted in the classification of these into three distinct groups (66). Except for one isolate of Whetzelinia sclerotiorum, the isolates analyzed were classified into three distinct groups; S.minor, S.trifoliorum and S.sclerotiorum.

Isozyme analysis has also been used to identify teliospores of the pathogen Tilletia indica without conducting long-term pathogenicity tests (13). Single-teliospore cultures of T.indica were examined by horizontal gel electrophoresis. The high number of alleles in common among isolates of T.indica permited their differentiation from those of T.barclayana the causal agent of kernel smut of rice.

Gill and Powell (37) determined that electrophoretic protein patterns of races A-1 to A-8 of Phytophthora

fragarie were nearly identical to each other, regardless of the locality or host from which the fungus was collected.

Matsuyama and Kosaka (45) determined that Pyricularia oryzae isolates from different sources could be divided into two major groups on the basis of soluble protein patterns and peroxidase zymograms, with no significant correlation with geographical distribution of the isolates. Based on pathogenicity criteria the authors found a correlation between three types of non-specific esterase zymograms and the three pathogenicity groups.

Electrophoretically detectable variation in the fungus Neurospora intermedia has been surveyed among isolates from natural populations in Malaya Papua, Australia and Florida (57). Results revealed a high level of genetic variation, mostly at the level of local populations.

Enzymatic polymorphism in strains of Colletorichum gleosporoides from Ivory Cost (34) revealed a great heterogeneity in the species. Enzymatic analysis showed that genetic structure of the population was not well defined.

Isozyme studies on the origin and evolution of Puccinia graminis f.sp.tritici in Australia, agreed with virulence studies in confirming the suggestion that most of the major changes in the wheat stem pathogen of Australia have resulted from overseas introductions (18).

Burdon et al. (17) studied isozyme uniformity and virulence variation in Puccinia graminis and P.recondita in Australia and did not detect any variation within either

pathogen species in the isozyme phenotypes of eleven different enzyme systems. Despite pronounced variation in virulence in both fungi, no variation was detected in the isozyme phenotypes among isolates of either pathogen. No polymorphism was found within the 12 isozymes detected in P.graminis or the 13 detected in P.recondita.

The use of isozyme analysis has been proposed to separate aggressive and non-aggressive isolates of Ceratocystis ulmi. Bernier et al. (10) studied seven enzyme systems in 15 isolates collected from various locations, and reported that isozymes were good biochemical markers for aggressiveness of C.ulmi isolates.

Alfenas et al. (2) studied isozyme and protein patterns in isolates of Cryphonectria cubensis differing in virulence. Results revealed differences and similarities among isolates with respect to their isozyme and protein patterns. Differences in the genotype affecting virulence were not associated with corresponding changes in protein patterns.

Newman (50) found no correlation between race of Rhyncosporium secalis among 26 isolates of the fungus and the isozyme patterns of their esterase, peroxidase, acid and alkaline phosphatase, glucosidase and galactosidase enzyme systems.

Burdon and Roelfs (19) detected marked differences in isozyme diversity and relative levels of isozyme and virulence diversity between asexual populations of Puccinia

graminis and P.recondita . In populations of P.recondita, diversity in virulence contrasted sharply with the low level of isozymic diversity, while in P.graminis nine different isozyme phenotypes were found. No isozymic differences were detected between isolates of the same race.

Later studies on the effect of sexual and asexual reproduction on the isozymes in populations of P.graminis (20) revealed a complex association of isozyme and virulence phenotypes in the asexual population, while no association was detected between individual isozyme alleles and virulence genes in the sexual population.

In order to investigate the potential role of sexual recombination in genetic variability of the fungus Magnaporthe grisea (teleomorph of Pyricularia oryzae), Leung and Williams (43) studied enzyme polymorphism among different geographic isolates. Results revealed that in contrast to the high degree of genetic diversity conditioning pathogenicity, relatively little variability was detected from the electrophoretic analyses.

Bonde et al (12) found no differences in isozyme patterns among isolates of the rust soybean pathogen Prakopsora pachyrhizi from Asia and Australia or the New World.

Isozymes of peroxidase, tetrazolium oxidase and certain protein bands, can be used to distinguish the two races of Ophiostoma ulmi the causal agent of Dutch elm disease (40). Electrophoretic separation of intramycelial peroxidase separated aggressive isolates from non-aggressive ones.

Tooley et al. (61) studied mating type, race composition, nuclear DNA content and enzyme analysis of Peruvian isolates of Phytophthora infestans, and determined that all strains were very similar to U.S and European isolates. These results strongly suggested a common ancestry for Peruvian, U.S and European populations of the potato pathogen.

Otrosina and Cobb (51) studied isozyme variation among 26 isolates representing the three varieties of Leptographium wageneri. Seven of the ten enzyme systems tested showed only one electrophoretic form. The data from the three polymorphic enzymes supported the concept of three taxonomic varieties. Zambino and Harrington (67) later determined isozyme variation of 76 isolates representing the three taxonomic varieties of L.wageneri. Results revealed 14 combinations of electromorphs detected among the 76 isolates. Each type was found in only one variety of L.wageneri. Analysis of the results also supported the division of the species into three taxa.

Studies to better understand the pathogenic variation present in C. lindemuthianum, the causal agent of anthracnose of beans, have been concentrated on breeding for resistance and to a lesser extent on the genetic causes of its variation (4, 7, 11, 29, 32, 33, 38).

Much of the research with P. griseola, causal agent of angular leaf spot (ALS) on beans, has been to identify better sources of resistance to the pathogen. Numerous

studies confirmed pathogenic variation in P.griseola (3, 23, 30, 31, 44, 55, 63) but there are no reports of studies dealing with enzymes as genetic markers. Correa (31) studied isozyme variation present in a number of Latin American and African isolates of P.griseola. He determined that only two isozyme patterns were useful for the best resolved enzymes: catalase, esterase, leucine aminopeptidase, and adenylate kinase. Results revealed that all Latin American isolates exhibited both pattern 1 and pattern 2, while African isolates exhibited only pattern 1. Also, the author suggested the association of pattern 1 with the Andean large-seed bean types, and pattern 2 with the Mesoamerican small-seeded bean types. The close association between isozyme patterns in the pathogen and seed size which is associated with centers of domestication in beans suggested a possible coevolution of the pathogen with its host.

In summary few reports show a relationship between isozymes and variable degree of pathogenicity. These include Phytophthora fragarie (37), Pyricularia oryzae (45), asexual populations of Puccinia graminis (20) and Ophiostoma ulmi (40).

CHAPTER I

CHARACTERIZATION OF Phaeoisariopsis griseola (Sacc.) Ferr. BY ISOZYME ANALYSIS

MATERIALS AND METHODS

Collection and isolation of fungal strains

Fifty-five isolates of Phaeoisariopsis griseola from Latin American countries (Argentina, Brazil, Colombia, Costa Rica, Dominican Republic, Mexico, Nicaragua,and Puerto Rico), African countries (Burundi, Kenya, Malawi, Rwanda, Tanzania, Uganda, and Zaire) and one from the United States were studied. Identification and origin of isolates are indicated in Table 1 .

Most of the Latin American isolates and some African isolates were obtained from the CIAT bean pathology collection. Most African, some Latin American and the U.S. isolates were isolated at Michigan State University from samples collected in the mentioned countries (30, 31). All cultures were purified using monospore transfers and cultures were mantained on V-8 agar medium (125 mL V-8 juice, 15 g agar, 2.6 g calcium carbonate, 1000 mL distilled water) and stored at 4°C.

Pathogenic races were determined by inoculating a group of eight differential bean varieties (31). In this way fourteen different pathotypes were identified.

Table 1. Isolates of Phaeoisariopsis griseola obtained from Latin America and Africa.

Latin America		Africa	
Isolate designation	Isolate origin	Isolate designation	Isolate origin
Argentina		Burundi	
Arg 1	La Cocha	Bur 1	Gitega
Arg 3	Ceibalito	Bur 2	Gitega
Brazil		Bur 3	Kamara
Bra 1	Sao Bento	Kenya	
Bra 2	Sao Bento	ken 1	Thika
Bra 3	Caruaru	Ken 3	Kabete
Bra 4	Goiania	Malawi	
Bra 5	Capivara	Mal 2	(Unknown)
Bra 6	(Unknown)		
Colombia		Mal 4	Matapwata
Col 1	Cauca	Mal 5	Riphondo
Col 2	Cauca	Mal 7	Dedza
Col 4	Antioquia	Mal 8	Muera
Col 5	Valle	Mal 9	Muera
Col 6	Cauca	Mal 11	Muera
Col 7	Quindio	Rwanda	
Col 8	Cauca	Rwa 1	Rubenheri
Col 9	Valle	Rwa 2	Rubenheri
Col 10	Antioquia	Rwa 3	Butawe
Col 11	Valle	Tanzania	
Col 12	Cauca	Tan 1	Magamba
Costa Rica		Tan 2	Milungui
Cos 1	Esparza	Tan 3	Arusha
Cos 2	Fabio Baudrit	Tan 4	Arusha
Dominican Republic		Tan 5	Morning site
Dom 1	Unknown		
Guatemala		Uganda	
Gua 1	Jutiapa	Uga 1	Kiabahinga
Gua 2	Jutiapa	Uga 2	Kachwekano
Gua 3	Escuintla	Uga 3	Kamuganguzi
Gua 5	Cuyuta	Zaire	
Mexico		Zar 1	Mulungu
Mex 1	Tepame		
Nicaragua			
Nic 1	Carazo		
Puerto Rico			
Pur 2	Isabela		
Pur 4	Isabela		

Low pathogenicity was found in pathotypes 11 to 14, while the highest level of pathogenicity was found in pathotype 1(Table 2).

Isolates from each the 14 proposed pathotypes were used in this study.

Pathogen growth and extraction

Cultures of all 55 isolates were grown in replicate on V-8 medium and incubated at 24°C for 10-14 days.

For electrophoresis, mycelial disks of 4 mm diameter were excised from actively growing cultures and aseptically transferred into five 125-mL Erlenmeyer flasks containing 25 mL of liquid modified Fries medium (30 g sucrose, 5.0 g ammonium tartrate, 0.1 g NaCl, 0.13 g $CaCl_2 2H_2O$, 10 g yeast extract, 1000 mL distilled water). The cultures were incubated at room temperature (24°C) on a shaker. Mycelial growth was harvested after 14 days of incubation. Harvested mycelia were vacuum filtered on nylon mesh and rinsed with sterile distilled water to remove the culture medium. Mycelial mats were blotted dry with sterile paper toweling and stored at -20°C.

Isozyme analysis

Protein extraction

Procedures for enzyme extraction were according to Correa (31). Samples of 5.0 mg of dried frozen mycelium

Table 2. Proposed pathogenicity groups in Phaeoisariopsis griseola. Sensu Correa (31).

	Pathogenicity group[a]													
Host cultivar	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Montcalm	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Seafarer	S	S	S	S	S	S	S	S	S	S	S	R	R	R
BAT 332	S	S	S	S	S	R	S	S	S	S	S	S	R	R
Pompadour Checa	S	S	S	S	R	S	S	S	S	R	R	R	R	R
G 5686	S	S	S	R	S	S	R	R	S	R	R	R	S	R
Cornell 49-242	S	S	S	S	R	S	R	S	R	R	R	R	R	R
A 339	S	R	R	S	R	R	S	R	R	R	S	S	R	R
BAT 1647	S	S	R	R	S	R	R	R	R	R	R	R	R	R

[a] S: Susceptible, 1% or more leaf area covered by P.griseola lesions

R: Resistant, < 1% leaf area covered by lesions

were combined with 1 mL of extraction buffer (170 g sucrose, 1 g ascorbic acid, 1 g cysteine hydrochloride, 1000 mL 0.1 M tris-citrate buffer ph 8.7) in the presence of acid-washed sand. The mixture was then triturated using a prechilled small mortar and pestle. Triturate was centrifuged in 1.5 mL eppendorf microcentrifuge tubes at 20,000 g for 20 minutes.

Crude extract was adsorbed onto paper chromatography wicks (3x15 mm) and wicks were kept in the freezer overnight. Temperature during extraction and centrifugation was at 4°C or less to avoid denaturation of proteins.

Electrophoresis

Horizontal gel electrophoresis and staining for specific enzymes were performed according to Correa (31) and Weeden (64).

Only the four enzyme systems, esterase, catalase, leucine aminopeptidase, and adenylate kinase, that showed polymorphism in previous work (31) were selected for the present study. Electrophoresis was conducted using three Weeden standard buffers in combination with the four enzyme systems used in Correa's work.

System I, a discontinuous buffer system, consisted of an electrode buffer containing 0.03 M lithium hydroxide monohydrate and 0.19 M boric acid, pH 8.1 and a gel buffer containing 1 part of electrode buffer to 9 parts of 0.05 M tris-citrate buffer pH 8.4.

System II, a continuous buffer, consisted of an electrode buffer containing 0.065M L-histidine and 1.62 g citric acid monohydrate, pH 6.5 and a gel buffer containing 1 part of electrode buffer to 3 parts of distilled water.

System III, also a continuous buffer system, consisted of an electrode buffer containing 0.04 M citric acid.H_2O with the pH adjusted to 6.1 with N-(3-aminopropyl morpholine) and a gel buffer that was a 1:10 dilution of the electrode buffer.

Gels were prepared to contain 330 mL of the gel buffer and 33 g of potato starch (Sigma Chemical Co.). The heated (boiling point) and degassed starch solution was poured into a plexiglass frame of dimensions: 22cmx22cmx1.5 cm. The gels were allowed to cool for two hours, and left at room temperature overnight. Gels were refrigerated for at least 1 hour before samples were loaded.

Twenty-four paper wicks containing the extracts (8 samples x 3 replications) were inserted into a slice made in the starch gel at 5 cm from the cathodal side of the gel. After loading the samples, the gel was placed in a refrigerator at 2°C and the gel was run at 50 mA for systems I and III, and at 25 mA for system II. After 20 minutes the wicks were removed and electrophoresis continued for 4 hours at 45 mA for systems I and III and for 3 hours at 25 mA for system II.

Staining

After electrophoresis each gel was sliced into five

equal horizontal slabs using plastic spacers and a fine nylon fishing thread. The resulting slabs were immersed in stain solutions specific for esterase, catalase, leucine aminopeptidase, and adenylate kinase (Appendix A). Gels were kept in the dark during staining to prevent excess background staining and to protect light sensitive reagents.

Scoring and interpretation of zymograms

Appearance, position and intensity of the bands were continuously monitored for each of the buffer-enzyme system combinations. The evaluation method was based on the presence or absence of bands, and their position and intensity.

Numbering of isozyme bands was standarizaed by giving the lower number to the most anodal band and larger numbers in the cathodal direction to the origin. Diagrams were drawn for each of the enzyme-buffer system combinations; photographs were made for a permanent record.

Pictures and diagrams of the different isozyme patterns were compared and monomorphism or polymorphism determined in each one of the detected enzyme loci.

RESULTS

Isozyme analysis

Three enzyme systems were detected in populations of P. griseola: esterase, catalase and leucine aminopeptidase (Figure 1). Differences in isozyme patterns and resolution of bands were observed when different buffer systems were used to resolve the different enzyme loci. Buffer system I was the best in resolving EST and CAT zymograms, whereas system II was the best for LAP. Analysis of the different isozyme patterns revealed 26 bands of enzymatic activity, 19 of them present in EST, 5 in CAT, and 2 in LAP (Figure2). The isozyme patterns of these enzymes are summarized in Table 3.

Esterase

Zymograms of EST allowed the detection of more polymorphism in populations of P. griseola than that reported by Correa (31). EST appeared to be resolved into 19, 14, and 10 bands with buffer systems I, II, and III respectively.

The effect of buffer system on the detection of EST and other enzymes was measured as a combination of resolution, number of bands and amount of detected polymorphism. Combinations of the above criteria were better provided by buffer system I. Bands appeared to be sharper and more separated with this buffer than with buffers II and III.

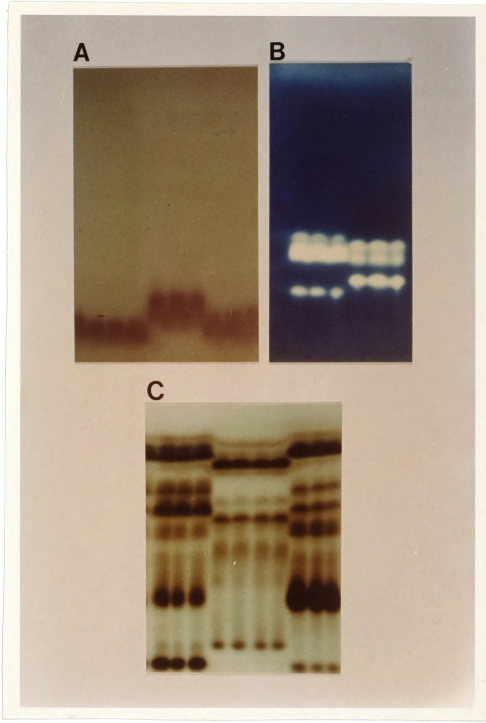

Figure 1. Enzyme loci detected in 55 isolates of Phaeoisariopsis griseola from Larin America, Africa and USA. Bands were detected with buffer system I (for B:catalase and C:esterase) and buffer system III (A:leucine aminopeptidase).

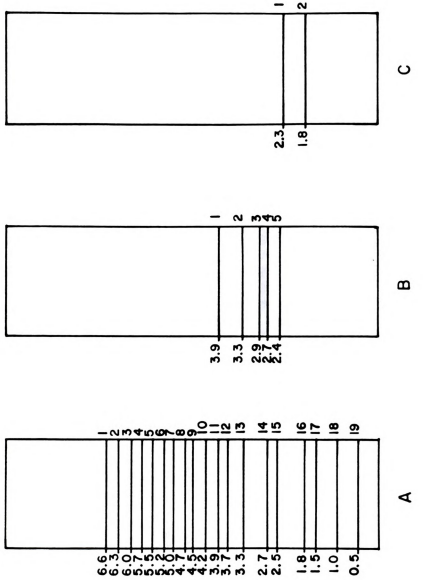

Figure 2. Total number of bands observed in each of the detected enzymes; A: esterase; B: catalase; and C: leucine aminopeptidase. Bands numbering on the right, bands frequency on the left.

Table 3. Electrophoretic patterns for three enzymes detected in 55 isolates of P. griseola from Latin America, Africa, and USA.

Origin of isolates	Isolates	Electrophoretic patterns EST	CAT	LAP
Latin America				
Argentina	Arg 1	8	3	2
	Arg 3	1	1	1
Brazil	Bra 1, 3	2	2	1
	Bra 2, 5	2	-	1
	Bra 4	2	4	1
	Bar 6	2	5	2
Colombia	Col 7, 8, 9	1	1	1
	Col 1, 2, 11	2	3	2
	Col 4	1	3	1
	Col 5	4	2	2
	Col 6	6	3	2
	Col 10	7	3	1
	Col 12	2	5	2
Costa Rica	Cos 1	2	2	2
	Cos 2	4	2	2
Dominican Republic	Dom 1	2	1	1
Guatemala	Gua 1, 2, 3	2	2	2
	Guat 4	4	2	2
Mexico	Mex 1	5	4	1
Nicaragua	Nic 1	1	4	1
Puerto Rico	Pur 2, 4	3	1	1
Africa				
Burundi	Bur 1, 2, 3	1	1	1
Kenya	Ken 1, 2	1	1	1
Malawi	Mal 2, 4, 5	1	1	1
	7, 8, 9	1	1	1
	11	1	1	1
Rwanda	Rwa 1, 2, 3	1	1	1
Tanzania	Tan 1, 2, 3	1	1	1
	4, 5	1	1	1
Uganda	Uga 1, 2, 3	1	1	1
Zaire	Zar 1	1	1	1
USA (Michigan)	Mich 5	1	3	1

EST: Esterase
CAT: Catala
LAP: Leucine aminopeptidase

Eight different isozyme patterns were detected with the EST-system I combination (Figure 3). Among the 55 isolates tested, all African, six Latin American, and the American isolate exhibited pattern 1. Only Latin American isolates exhibited patterns 2 to 8. Patterns 1 and 2 were found in 31 and 15 isolates, respectively while patterns 3 to 8 were found in three to one isolates.

Differences in banding patterns were detected between African and Latin American populations of P. griseola. More variation was observed among Latin American than among African isolates.

Two of the 19 bands detected with buffer system I (Figure 2A) were exhibited by a large number of isolates . Band 6 was found in 90% of the isolates, while band 9 was present in 89%. Incidence of the other bands ranged from 2 to 67%. Bands with a frequency of less than 5% were present in patterns containing only one isolate.

Number of bands in the isozyme patterns ranged from 3 to 8. Eight bands were present in patterns 1, 5, and 7. Band 5 was common to all three patterns. Patterns 2 and 6 exhibited six bands with bands 2, 6, and 8 common to both patterns. Among all isozyme patterns, pattern 5 present in the Mexican isolate, was the only one exhibiting four unique bands 4, 10, 14, and 16.

Catalase

Buffer systems II and III allowed the detection of four

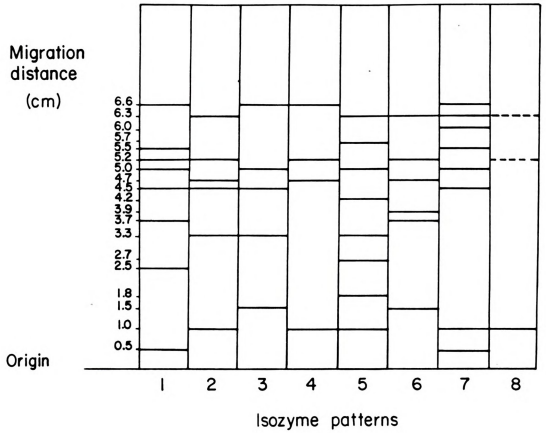

Figure 3. Esterase isozyme patterns detected in 55 isolates of Phaeoisariopsis griseola from Latin America, Africa, and USA. Bands were detected with buffer system I.

different catalase bands, while buffer system I revealed five. All isolates were distributed in five different isozyme patterns with buffer systems I and III, while buffer system II grouped these in four. Pattern 1 in all three buffer systems contained most of the African isolates. Two African isolates, Malawi 8 and Tanzania 2, exhibited a different pattern with buffers II and III.

Buffer system I was the best, showing polymorphism, clear resolution, and different numbers of the bands. Five different isozyme patterns were detected with this buffer system (Figure 4). Pattern 1 contained all the African isolates and seven Latin American isolates. Patterns 2 to 5 contained only Latin American isolates.

Results of the total number of bands resolved with buffer system I indicated bands 1 and 2 as the most frequent among P. griseola isolates (Figure 2B). Band 2 was present in all the isolates, while band 1 was observed in 93% of the isolates. Band 5 exhibited the lowest frequency among the isolates.

As observed with EST, the CAT-system I was monomorphic for the African isolates but polymorphic for the Latin American isolates.

Leucine aminopeptidase

Zymograms of LAP in all three buffer systems indicated the presence of a single major band with two mobility variants. Differences between buffer systems were only detected

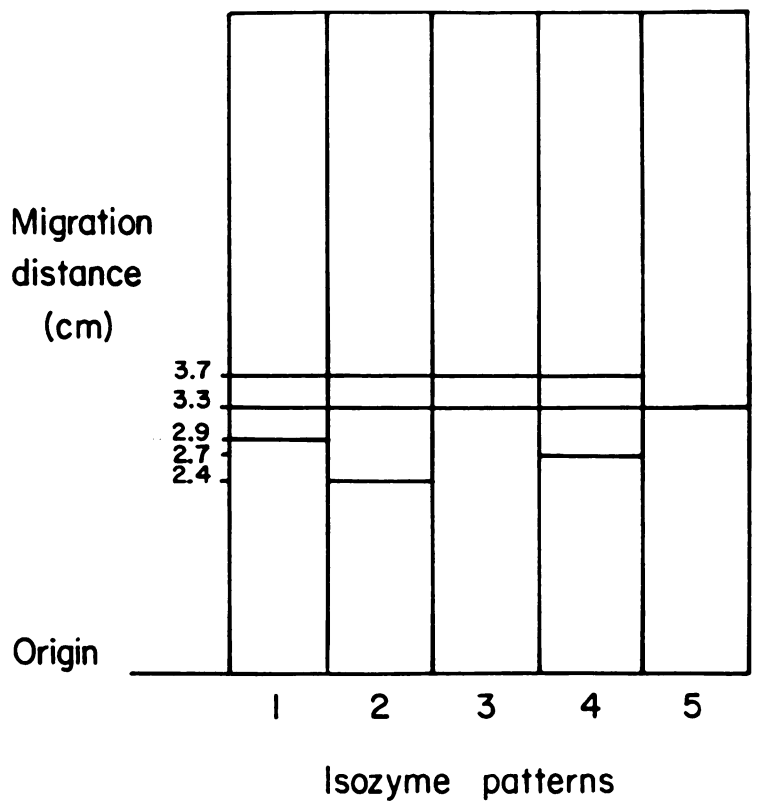

Figure 4. Catalase isozyme patterns detected in 55 isolates of Phaeoisariopsis griseola from Latin America, Africa, and USA. Bands were resolved with buffer system I.

in the migration of the bands. Bands resolved with buffer system I migrated further from the origin than those resolved with systems II and III. Buffer system II produced the best resolution of the two LAP isozymes patterns (Figure 5). Pattern 1 was exhibited by all African, fifteen Latin American and the Noth American isolates. Pattern 2 was only detected in the Latin American isolates.

LAP enzyme exhibited less complex isozyme patterns and a lower level of polymorphism as compared with EST and CAT enzymes. Clustering of P. griseola isolates into two LAP isozyme patterns agreed with results previously presented by Correa (31), where African isolates exhibited only one pattern, while Latin American isolates exhibited both patterns. Although EST, CAT, and LAP isozyme patterns exhibited clustering of all African isolates in a single group, Latin American isolates exhibited more than one isozyme pattern for all three enzymes.

Genetic interpretation of EST, CAT, and LAP zymograms was not possible in the present study, since analyses were performed with the asexual stage of the fungus. Variation due either to multiple loci coding for different proteins with the same enzyme activity, or for multiple alleles functioning at a single locus could not be determined.

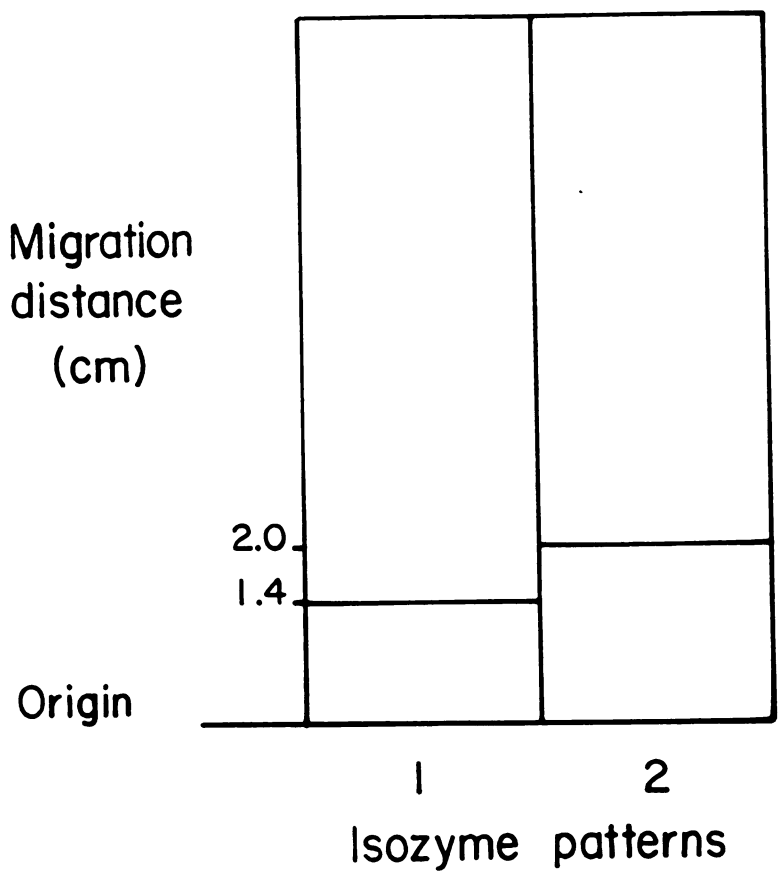

Figure 5. Leucine aminopeptidase isozyme patterns detected in 55 isolates of Phaeoisariopsis griseola from Latin America, Africa, and USA. Bands were resolved with buffer system I.

DISCUSSION

Electrophoretic analyses of Phaeoisariopsis griseola isolates revealed both polymorphism and monomorphism among populations of the fungus. Latin American isolates were polymorphic for EST , CAT, and LAP enzyme loci, while those from African countries were monomorphic for these enzymes.

Variability in isozyme patterns was detected with three different resolving buffers. Buffer system I was selected for EST and CAT enzymes, while buffer system II selected for LAP enzyme.

Differences in banding pattern, number and resolution of bands could be interpreted as a product of system/enzyme interaction. Buffer system I, used as a discontinuous buffer, normally resulted in sharper zones of activity because of the effect of the front (the interface between the two buffer systems) as it moved along the gel. Buffer system I, with a high ionic strength, gave sharper separation of bands with EST and CAT. LAP enzyme system showed a better interaction with low ionic strength buffers. Thus, buffer systems II and III allowed a better resolution of bands with LAP.

Our results agree with those of Correa (31) as to the presence of monomorphism in African isolates of P. griseola. However significant areas of disagreement were found with respect to degrees of polymorphism found in isolates from

Latin America.

Correa reported the separation of Latin American isolates into two groups according to the isozyme patterns for EST, CAT, LAP and AdK. Results of the present study indicated more than two patterns for EST and CAT, two for LAP and absence of enzymatic activity for AdK enzyme.

In Correa's work (31), EST zymograms revealed two isozyme patterns with buffer system III for all isolates of P. griseola. Results of the present study indicated a total of eight different patterns. Patterns 1, 2, and 3 were found in African isolates, which indicates a higher level of polymorphism than that observed by Correa among African populations.

Comparison of EST zymograms resolved with buffer system III to those resolved with buffer system I, revealed marked differences in resolution and sharpness of the bands. Bands resolved with buffer system III appeared closer, less sharp and fewer in number as compared with those resolved with buffer system I. Interpretation of zymograms became more difficult in zymograms of system III than in those of system I. Thus, differences related to criteria used in scoring and interpretation of zymograms may be reasons for the disagreement between Correa's work and the present study.

The data presented here support the general conclusion as to the presence of variation between the genetic structure of the African and Latin American populations of P. griseola. The diversity of the Latin American population

was greater than that of the African population.

Pathogenicity groups of African and Latin American isolates were not correlated to isozyme patterns. The African isolates of P. griseola showed a much greater diversity of pathogenicity groups while being isozymically much more uniform. Although Latin American isolates showed a great diversity in pathogenicity groups and isozyme patterns, pathogenicity groups were not related to isozyme phenotypes. The number of race determining genes varies greatly between pathotypes and in many cases (especially in physiological races) such genes constitute only a very small part of the pathogen's genome (65). Lack of correspondence between pathogenicity groups and isozyme patterns in P. griseola, may indicate the insufficiency of the electrophoretic analysis to detect gene affecting race variability.

Correa (31) proposed an association between electrophoretic patterns of ALS isolates and bean seed types from where they were isolated. He suggested pattern 1 to be associated with isolates infecting large-seeded bean types, while pattern 2 was associated with isolates infecting small-seeded bean types.

Results presented here suggest that pattern 1 of EST, CAT and LAP has a common ancestry for African and Latin American populations of P. griseola. High incidence of EST bands 6 and 9 and CAT bands 1 and 2 in the majority of the isolates could be considered as common alleles of these two geographic populations. Bands showing a low frequency among

isolates might be considered as new products of recombination or result of possible mutations.

According to Kaplan (41) by the early 1500's beans had been domesticated in South America for several thousand years. Samples of these beans (probably large-seeded) could have been introduced to Africa along with associated seed-borne pathogens like P. griseola. In this way, a portion of pattern 1 pathogen type could have been carried to Africa and evolved with large-seeded bean types, which are the most prevalent types under production in that continent. Many reports (9, 36, 41, 56) agree that the common bean originated in the Americas where the diversity among the wild and related species is greatest. Middle America and South America have been hypothesized as independent domestication centers for the common bean; these two areas, considered the most important, have led to extensive cultivation of small-seeded and large-seeded bean cultivars, respectively.

It is probable that genes of the bean cultivars from Andean and Middle American gene pools have interacted with P. griseola genes resulting in changes of the pathogen's isozyme patterns. If pattern 1 is considered as a product of the coevolution of genes from Andean bean cultivars and those of P. griseola, this remained stable prior to the time when new resistant genes were introduced into this domestication center through breeding. Once foreign resistant genes were transferred into native cultivars, correlations between zymograms and geographical distribution of patterns

may have tended to disappear. In this way the generation of new isozyme patterns might have occurred in the pathogen populations from South America.

Matsuyama and Kosaka (45) reported two groups of isolates of the rice blast fungus Pyricularia oryzae which were distinguished by patterns in the soluble protein and peroxidase zymograms. However these characters were not significantly correlated with the geographical distribution of the isolates or their pathogenicity. Lack of correspondence between zymograms and geographical distribution of isolates, was considered as a result of the interaction of foreign resistant genes (transferred into Japanese rice varieties) with genes of the rice pathogen.

If an association between pattern 1 in P. griseola and large-seeded bean types exists, this probably originated in South America and later spread to Middle America and Africa.

Intense breeding efforts in areas of South America might have resulted in the introduction of foreign resistance genes into bean cultivars, which induced changes in the genetic structure of P. griseola.

Lack of sexual reproduction in the pathogen, environmental homogeneity, and type and number of propagules colonizing a site, have all been correlated with low electrophoretic variability in fungal populations. Homogeneity present in the African population of P. griseola may be the result of the introduction of only a few isolates of the ancestral population. These isolates, might have

carried a fraction of the genetic variability that exists in the species.

Lack of major progress in breeding for resistance to P. griseola in Africa may be a contributing factor towards maintaining a stable genetic structure in the population of P. griseola. Correlation between zymograms and geographical distribution of pattern 1 in Africa could be compromised as foreign resistant genes are used in breeding programs.

Cropping systems in Africa, specially Malawi, have maintained a special environment to the pathogen. Mixtures of landraces in Malawi (mostly large-seeded bean types) and other African countries, could be considered as evolutionary barriers to the pathogen.

In summary, pattern 1 in EST, CAT, and LAP enzymes is suggested to represent the initial genotype of P. griseola isolates. Pattern 1 might in the future exhibit different evolutionary paths in the American and African continents.

The nature of the homogeneity and heterogeneity among African and Latin American populations respectively, of P.griseola does not have a genetic explanation at this point, since current research dealtg only with the asexual stage of the fungus.

Although the sexual stage of P.griseola is unknown, and artificial hybridization has been difficult until now, new studies are needed to better understand the genetics of this pathogen. Results of this study suggests that such efforts concentrate on those pathotypes exhibiting pattern 1.

Electrophoretic analysis of the progenies of crosses between isolates exhibiting pattern 1, could elucidate the nature of the genetic variability present in this fungus.

CHAPTER II

CHARACTERIZATION OF *Colletotrichum lindemuthianum* (Sacc. and Magn.) ISOLATES BY ISOZYME ANALYSIS

MATERIALS AND METHODS

Collection and isolation of fungal specimens

Eighteen monosporic isolates of *Colletotrichum lindemuthianum* included nine Colombian isolates from the main bean growing areas in Colombia, and nine European races already characterized by Drijfhout (32) were used in this study. Isolates were obtained from the CIAT bean pathology collection. The identification and origin of specimens are indicated in Table 4.

Pathogen isolates were grown for 10 days on PDA medium (39 g Difco dehydrated potato dextrose agar medium, and 1000 mL distilled water) and then stored at 4°C until needed.

Race characterization

Seed source

A group of nine Colombian isolates was tested for pathogenicity on the twelve selected bean genotypes proposed for the identification of *C.lindemuthianum* races by Drijfhout (32) (Table 5).

Table 4. Identification and origin of *Colletotrichum lindemuthianum* isolates

Isolate	Origin	Variety from which isolated
Colombia		
CL 1	Pasto-Nariño	-
CL 2	Palmira-Valle del Cauca	ICA-L-24
CL 3	La cumbre-Valle del Cauca	-
CL 4	La Selva-Antioquia	Diacol-Catio
CL 5	Guacas-Cauca	-
CL 6	El Refugio-Cauca	PI165426
CL 7	La Selva-Antioquia	G02858
CL 8	La Selva-Antioquia	BAT 93
CL 9	La Selva-Antioquia	-
Europe		
α-Brazil	Drijfhout	-
C-236	Drijfhout	-
Delta	Drijfhout	-
Gamma	Drijfhout	-
Iota	Drijfhout	-
Kappa	Drijfhout	-
42-80 K	Fouilloux	-
Lambda	Drijfhout	-
Epsilon Kenya	Drijfhout	-

-: not known

Table 5. Proposed standard range of differentials for the identification of races of Colletorichum lindemuthianum according to E. Drijfhout, IVT, Holland (32).

Resistance group	Differential variety	Reaction to Race									
		Epsilon Kenya	Alpha	Gamma	Alpha Brazil	Delta	Beta	kappa	C 236	Lambda	Iota
1	Michelite	+	+	+	+	+	+	+	+	+	+
2	Auguille Vert	+	+	-	+	+	+	+	+	+	+
3	Mich. D. R. Kidney	-	-	+	-	+	+	+	-	+	+
4	Sanilac	-	-	-	-	+	+	+	+	+	+
5	Perry Marrow	-	-	+	-	+	-	+	-	+	+
6	Coco a la Creme	-	-	+	-	-	+	-	+	+	+
7	P. I. 167399	-	+	-	+	-	+	-	-	+	-
8	P. I. 165426	+	-	-	+	-	-	-	+	-	-
9	Cornell 49242	-	-	-	-	-	-	+	+	-	+
10	Evolutie	-	-	-	-	-	-	-	+	-	+
11	Mexico 222	-	-	-	+	-	-	-	-	-	-
12	AB 136	-	-	-	-	-	-	-	-	-	-
Number of attacked differentials		3	3	4	5	5	6	6	7	7	8

+: susceptible; -: resistant

Inoculum preparation

Spores were increased on bean leaf PDA amended medium (PDA-bl). The PDA-bl was prepared by sterilizing healthy bean leaves by autoclaving for 20 minutes at 121°C and then placing the leaves onto the surface of solidified PDA plates. Ten drops of a C.lindemuthianum spore suspension were uniformly spread onto PDA-bl medium and the cultures incubated at 20°C for 7 days.

Spore suspensions were prepared by gently scraping the surface of profusely sporulated cultures with a spatula, and mixing the spores with water. Tween 20, a wetting agent, was added to the water at 0.1 % v/v. Spore concentration was adjusted to $1.2x10^6$ spores/mL using a hemacytometer (American Optical Co.).

Seven day-old greenhouse-grown seedlings of the twelve bean anthracnose differential genotypes (ten seedlings per variety) were inoculated with each of the Colombian isolates by gently spraying the spore suspension on the upper and lower leaf surfaces. Inoculated seedlings were then incubated in a mist chamber (± 90% relative humidity and ± 22°C) for 5 days. Physical separation between plants inoculated with different isolates was practiced to avoid possible cross contamination. Pathogenicity was evaluated 7 days after inoculation.

Plant reactions were scored as resistant (R), inter mediate (I) and susceptible (S) where R represented

those plants without visible symptoms or with a few scattered, small lesions on the midrib and occasionally on main veins; I represented those plants showing many small lesions scattered on the midrib and veins with collapse of the tissue, and S represented those plants showing many large lesions spread over the leaf blade. Plants rated S also showed many large coalesced lesions accompanied by tissue breakdown.

In order to compare the reactions of the twelve differential cultivars inoculated with the Colombian and European isolates (Table 5), plants with a resistant reaction were defined as (-), and those with intermediate and susceptible reactions as (+).

Isozyme analysis

Culture methods

Samples of C.lindemuthianum mycelia grown for different periods were electrophoretically assayed to determine the optimum growth period for detecting enzyme activity.

Actively growing mycelial plugs (0.4 mm diameter) were cut from colonies of iota, lambda, delta, and kappa races, and transferred to five 125 mL Erlenmeyer flasks each containing 30 mL of M2 medium (Modified Fries medium: 30 g sucrose; 50 g ammonium tartrate; 1.0 g KH_2PO_4; 1.0 g NH_4NO_3; 0.5 g $MgSO_4.7H_2O$; 0.1 g NaCl; 0.13 g $CaCl_2.2H_2O$; 1.0 g yeast extract and 1000 mL distilled water). Cultures were grown for 7, 14, and 21 days at room temperature (± 22°C), on a

reciprocal shaker. Tests were replicated five times. All samples were harvested at the same time by filtration. Mycelial mats were washed with sterile distilled water in a Buchner funnel, drained, and blotted with paper towel and then frozen at -20°C.

Once the best growth period was determined, cultures of the four European races were grown in three different media to determine the effect of medium on enzyme activity.

Actively growing mycelial plugs (0.4 mm) cut from colonies growing on PDA medium were transferred to 125 mL erlenmeyer flasks containing 30 mL of M1 (500 g peeled potatoes, 10 g dextrose, 1g NaCl, 1000 mL distilled water), M2 (Modified Fries medium) or M3 medium (10 g proteose peptone, 15 g dextrose, 0.25 g $MgSO_4.7H_2O$, 0.5 g K_2HPO_4, and 1 1000 mL distilled water). Cultures were grown for 14 days at room temperature on a reciprocal shaker. After 14 days cultures were harvested and processed as described above.

Protein extraction

Small portions (500 mg) of frozen samples were ground in a prechilled mortar with 1 mL of cool extraction buffer containing acid-washed sand as previously described. Triturated samples were centrifuged in 1.5 mL eppendorf microcentrifuge tubes at 20,000xg for 20 minutes and the resulting supernatant absorbed on 3x15 mm paper chromatographic wicks, and kept in the freezer overnight.

Electrophoresis

Horizontal starch gel electrophoresis was carried out

by using the methods previously described. Buffer systems I, II, and III described by Weeden (64) were used to resolve the enzymes Catalase(CAT), Esterase(EST), Leucine aminopeptidase(LAP), Malate dehydrogenase(MDH), Alcohol dehydrogenase(ADH), Glutamate dehydrogenase(GDH), Glucose phosphate isomerase(PGI), malic enzyme (ME), Phosphoglucomutase(PGM), Diaphorase(DIAP), Adenylate kinase(AdK), Shikimate dehydrogenase(SKDH), 6-phosphogluconate dehydrogenase(6-PGDH), Methyl umberiferyl esterase(Mu-EST), Peroxidase(PRX), Acid phosphatase(ACP), and Isocitrate dehydrogenase(IDH). Full names, abbreviations, and code numbers of the enzymes are indicated in Table 6.

After electrophoresis, gels were sliced and stained as described before. Protocols for staining are indicated in Appendix A.

Scoring and interpretation of zymograms

Isozyme patterns detected with the three buffer systems were scored as described previously for P.griseola.

Host gene pool determination

It has been postulated that domestication of the common bean took place independently in two different regions; the Mesoamerican, and the Andean regions (36).

This classification in two different gene pool has been proposed on the basis of seed size, where Mesoamerican cultivars are considered the small-seeded gene pool while the Andean cultivars are considered the large-seeded gene pool.

Table 6. Full name and code number of tested enzymes

Enzyme	Abbreviation	Enzyme code number
Acid phosphatase	ACP	3.1.3.2
Adenylate kinase	AdK	2.7.4.3
Alcohol dehydrogenase	ADH	1.1.1.1
Catalase	CAT	1.11.1.6
Diaphorase	DIAP	1.6.4.3
Esterase	EST	3.1.1.1
Glucose phosphate isomerase	PGI	5.3.1.9
Glutamate dehydrogenase	GDH	1.4.1.2
Isocitrate dehydrogenase	IDH	1.4.1.42
Leucine aminopeptidase	LAP	3.4.11.1
Malate dehydrogenase	MDH	1.1.1.37
Malic enzyme	ME	1.1.1.40
Methyl umberiferyl esterase	Mu-EST	-
Peroxidase	PRX	1.11.1.7
Phosphoglucomutase	PGM	2.7.5.1
6-phosphogluconate dehydrogenase	6-PGDH	1.1.1.44
Shikimate dehydrogenase	SKDH	1.1.1.25

Phaseolin, the major seed storage protein in common bean, and DIAP-enzyme present in roots, have been found to be two very useful criteria for the differentiation of the bean gene pools (64, 58). Six storage protein types have been identified in seed. The "S" and "B" phaseolins occur in varieties with smaller seeds than do "A", "C", "H", and "T" phaseolin (58). Sprecher (58) reported a strong association between DIAP-phenotypes and bean seed size. Results revealed six different isozyme patterns identified as fast, slow, unique, rare, null-1 and null-2 (Figure 6). Seeds showing the fast-phenotype were considerably larger than those exhibiting the slow-phenotype.

The gene pool identity of the bean cultivars used in race characterization of C.lindemuthianum was determined. The objective of this analysis was to determine if isozyme patterns present in populations of C.lindemuthianum were related to the gene pools present in the common bean.

Sample preparation

Seeds of the twelve bean anthracnose differential cultivars were planted in vermiculite. After seven days seedlings were removed and the roots washed with tap water. About 100 mg of healthy clean roots, was cut from each cultivar, and individually mixed with 0.2 mL of cool extraction buffer 0.1 M tris-maleate pH 8.0 (1.0 g tris per 80 mL distilled water, 10 mL glycerol, 10 g polyvinylpyrrolidone-40 pH adjust to 8.0 with maleic anhydride). Samples were then homogenized on prechilled porcelain spotting plates.

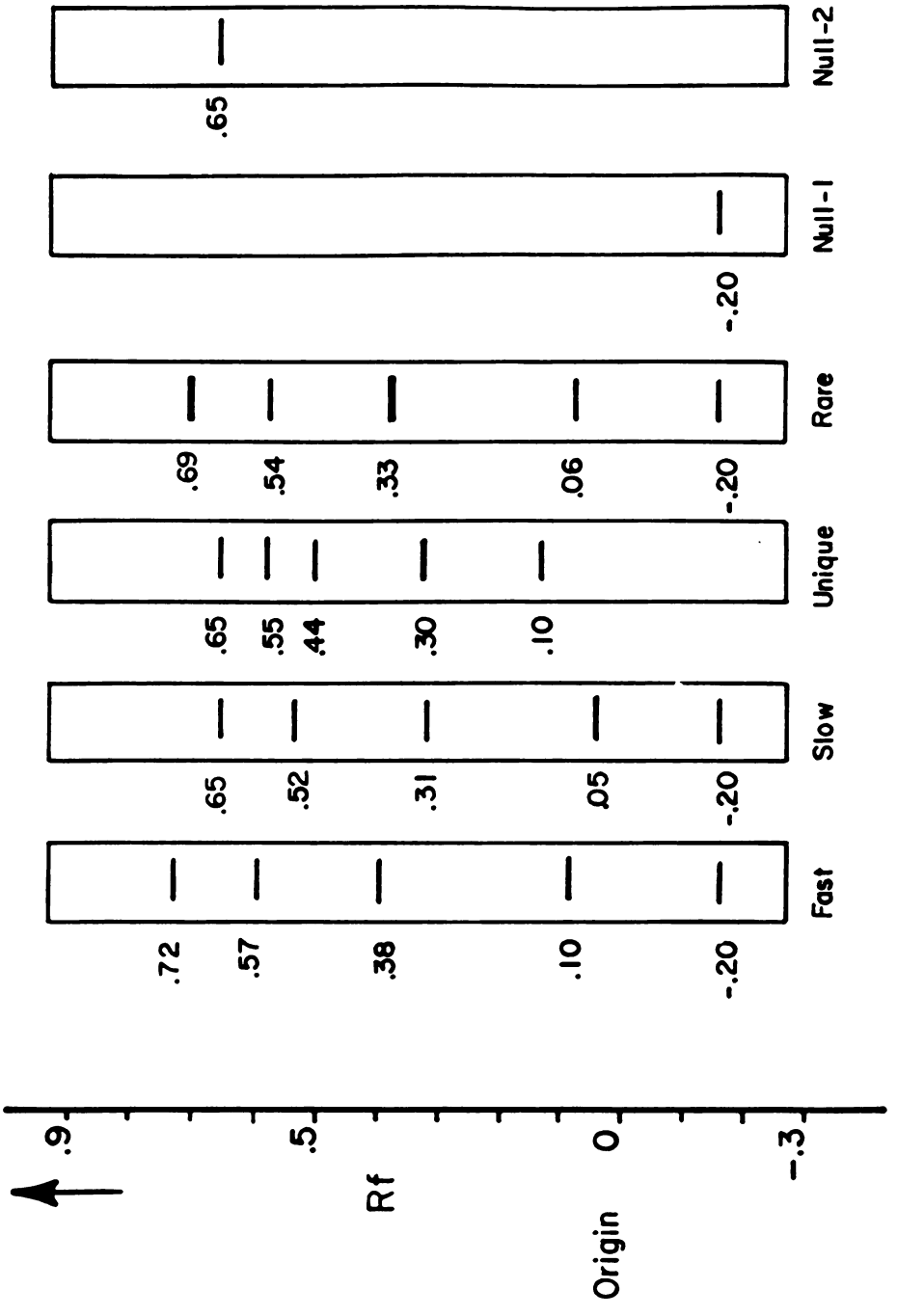

Figure 6. Diaphorase isozyme patterns present in the common bean. Source. Sprecher (58).

Crude extracts were adsorbed on paper chromatography wicks (4x5 mm) and kept in the freezer overnight.

Root tissues of Sanilac and Tendergreen cultivars were also analyzed and considered as control samples of known DIAP and phaseolin phenotypes.

For phaseolin analyses, seeds of the twelve anthracnose differential cultivars were soaked for 12-24 hours, and then a 2x2 mm portion of cotyledon was removed and individually homogenized in 0.2 mL of the 0.1 M tris-maleate extraction buffer.

Electrophoresis

Horizontal gel electrophoresis was carried out following procedures previously described. Gel preparation and electrophoresis buffer systems were those of Weeden's system I (64). Gels were run at 45 mA for 4 hours at 2°C.

Staining

After electrophoresis gels were sliced into five equal slabs and then stained for DIAP in case of root samples, and for phaseolin in case of seed samples. DIAP stain solution was the same as used previously. The phaseolin stain solution contained: 20 mL wash solution (100 mL methanol, 100 mL distilled water, 20 mL acetic acid) and 20 mg naphthol blue black. After gels were stained and bands resolved, isozyme patterns were determined for each of the bean cultivars.

Scoring of different DIAP phenotypes was according to Sprecher (58) (Figure 6). Electrophoretic variants were

designated as Fast (F), Slow (S), Intermediate (I) or Null (N) depending on their relative mobility or absence of activity. Phaseolin phenotypes were also classified according to relative mobility of the bands as fast or slow phenotypes. Fast phenotypes were indicative of the Andean gene pool and slow phenotypes as the Mesoamerican gene pool.

RESULTS

Race characterization

Reactions of the bean differential varieties to the Colombian and Delta European isolates of Colletotrichum lindemuthianum are summarized in Table 7. Results were compared to those reported by Drijfhout (32) for European races of the fungus. Colombian isolates appeared different from each other. The CL5 isolate exhibited a reaction spectrum identical to European Delta race. The CL4 and CL5 isolates appeared very similar, differing only in their pathogenicity to line P.I.167.399, which was susceptible to CL4 and resistant to CL5 isolate.

Michelite and Aiguille Vert were susceptible to all Colombian isolates, while Coco a la creme, Mexico 222, and AB 136 were resistant to the same isolates.

The results indicate the that five Colombian isolates were able to overcome the immunity of cultivar Cornell 49-242. Isolates CL1, 7, 8, and 9 were pathogenic on Cornell 49-242, carrier of the "ARE" gene, a source of resistance to large number of C. lindemuthianum races.

Different levels of pathogenicity were observed among isolates tested. The greatest being observed in isolates CL7 and CL8 followed by CL2.

Table 7. Disease reactions of standarized differentials to Colombian isolates of Colletotrichum lindemuthianum. Sensu Drifjhout (32).

Differential variety	Reaction to isolate									
	CL1[b]	CL2	CL3	CL4	CL5	CL6	CL7	CL8	CL9	D[a]
Michelite	+	+	+	+	+	+	+	+	+	+
Aiguille Vert	+	+	+	+	+	+	+	+	+	+
Mich.D.R.Kidney	+	+	+	+	+	+	+	-	-	+
Sanilac	-	+	-	+	+	+	+	+	+	+
Perry Marrow	+	+	-	+	+	+	+	-	-	+
Coco a la crem	-	-	-	-	-	-	-	-	-	-
P.I.167.399	+	-	-	+	-	+	+	+	+	-
P.I.165.426	-	+	+	-	-	+	+	+	-	-
Cornell 49.2	+	-	-	-	-	-	+	+	+	-
Evolutie	-	±	-	-	-	±	-	-	-	-
Mexico 22	-	-	-	-	-	-	-	-	-	-
AB 136	-	-	-	-	-	-	-	-	-	-

[a] European Delta race

[b] +: susceptible
-: resistant
±: susceptible and resistant

Isolates CL7 and CL8 were pathogenic on eight differential cultivars, while CL2 was pathogenic on seven. The lowest level of pathogenicity was exhibited by isolate CL3 from La Cumbre Valle del Cauca. In general, results revealed a wide pathogenic variation in Colombian C. lindemuthianum isolates.

Reactions of Perry Marrow, Michigan Dark Red Kidney, and Michelite cultivars to Colombian C. lindemuthianum isolates, were considered in order to determine the presence of Brazilian and Mexican races among these isolates (Tables 8 and 9). Brazilian and Mexican races were found in populations of C. lindemuthianum from Colombia (Table 10). Delta group was present in six isolates, Alpha and Brazilian II groups in two isolates, and Mexican II group in isolate CL3.

Isozyme analysis

Preliminary analysis

Effect of culture age and medium on the amount of detectable enzyme loci, was determined for four European races of C. lindemuthianum. Results revealed differences in isozyme patterns, number and resolution of bands.

Six enzyme loci, EST, CAT, LAP, PGI, PGM, and DIAP, demonstrated well resolved patterns and showed electrophoretic variation among C. lindemuthianum isolates.

Isozyme patterns for EST with buffer system I revealed a higher enzymatic activity in 7 day-old cultures compared

Table 8. Reactions of several host varieties to Brazilian races of Colletotrichum lindemuthianum (Oliari et al; Pio-Ribeiro and Chavez, S. R) (29).

Differential variety	Reaction to Race									
	Alpha group		Bra.I group		Bra.II, Mex.I groups		Mex.II group			Delta group
	BA-1	BA-2	BA-4	BA-5	BA-3	BA-9	BA-6	BA-7	BA-8	BA-10
Michelite	+	+	+	+	+	-	+	+	+	+
Mich. D. R. Kidney	-	-	-	-	-	-	+	+	+	+
Perry Marrow	-	-	+	+	-	-	-	-	-	+
Emerson 847	-	-	+	+	+	-	-	+	-	+
Ph.aborigenous 283	+	-	-	+	+	-	+	+	+	-
Costa Rica 1031	+	+	+	+	+	+	+	-	-	+

+: susceptible
-: resistant

Table 9. Reactions of several host varieties to American and Maxican races of Colletotrichum lindemuthianum. (Yerkes & Ortiz; Yerkes Jr.) (29)

Differential variety	Reaction to Race												
	Alpha group			Mexican groups									
				I							II		III
	MA-11	MA-12	MA-13	MA-1	MA-2	MA-3	MA-4	MA-5	MA-6	MA-7	MA-8	MA-9	MA-10
Michelite	+	+	-	-	-	-	-	-	-	+	-	-	-
Mich. D. R. Kidney	-	-	-	-	-	-	-	-	-	+	-	-	-
Perry Marrow	-	-	-	-	-	-	-	-	-	-	+	+	+
Negro 150	-	-	+	+	+	-	-	-	-	-	+	-	+
Negro 152	+	-	+	+	+	+	-	-	-	-	+	+	+
Amarillo 155	+	+	+	+	+	+	+	+	+	-	-	-	+
Bayo 164	+	-	+	-	-	-	-	-	+	-	-	-	-
Canario 101	-	-	-	-	-	-	-	-	-	+	-	-	-

+: susceptible
-: resistant

Table 10. Presence of Mexican and Brazilian races among Colombian isolates of *Colletotrichum lindemuthianum*.

Isolate	Differential variety			Classification	
	Michelite	Mic. D. R. Kidney	Perry Marrow	Group	Race
CL-1	+	+	+	Delta	BA-10
CL-2	+	+	+	Delta	BA-10
CL-3	+	+	-	Mex.II	MA-7 or BA-6, BA-8
CL-4	+	+	+	Delta	BA-10
CL-5	+	+	+	Delta	BA-10
CL-6	+	+	+	Delta	BA-10
CL-7	+	+	+	Delta	BA-10
CL-8	+	-	-	Alpha, Bra.II	MA-11, MA-13, BA-1, BA-3
CL-9	+	-	-	Alpha, Bra.II	MA_11, MA-13, BA-1, BA-3

+: susceptible

-: resistant

to 14 and 21 day-old (Figure 7). ALthough enzymatic activity was very high at 7 days, few bands were observed in samples at this age.

Fourteen days was the best age for analysis of fungal isolates for most of the enzyme loci. Although 21 day-old samples gave good enzyme resolution and polymorphism, 14 day-old samples revealed a larger number of bands. CAT and LAP enzymes showed little variation among isolates when different sample ages were used for electrophoretic analysis. Differences were detected in sharpness and number of resolved bands when different buffer systems were used. Buffer systems II and III resolved a larger number of bands for CAT, while system I allowed a better resolution of the bands. Systems II and III were better for resolution of LAP enzyme.

Results from experiments on media composition revealed differences in the amount of enzymatic activity (Figure 8). Greater mycelial growth was observed on samples growing on M1 medium. Cultures growing on M1 medium showed more enzymatic activity than those growing on M2 and M3 media. LAP enzymatic activity seemed to be higher in samples growing on M3 medium than on those growing on M1 and M2 media.

The lambda race exhibited more variation due to culture conditions than any other isolate.

In summary, optimum enzymatic activity, resolution and polymorphism were observed in samples grown for 14 days on

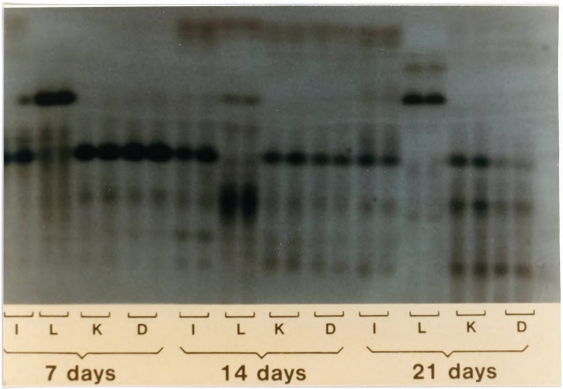

Figure 7. Differences in esterase isozyme patterns detected in 4 European races of Colletotrichum lindemuthianum harvested at three different age. Bands were resolved with buffer system I. I: iota; L: lambda; K: kappa; D: delta.

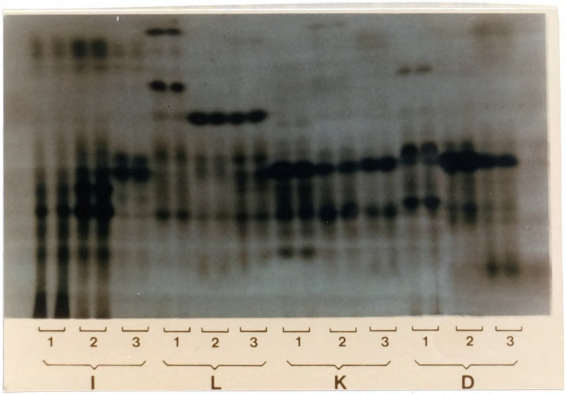

Figure 8. Differences in esterase isozyme patterns detected in 4 European races of Colletotrichum lindemuthianum when grown in three culture media. Bands were resolved with buffer system I. 1: M1 medium; 2: M2 medium; 3: M3 medium; I: iota; L: lambda; K: kappa; and D: delta.

M1 medium. This was true for all detected enzyme loci with the exception of LAP enzyme. Buffer system I was best for resolving EST, CAT, and DIAP enzymes, while system II was best for PGM, LAP, and PGI enzymes. A total of eighteen isolates, including nine Colombian and nine European races were then examined for the five detected enzyme loci.

Buffer systems I and II allowed the detection of a total of 32 sites of enzymatic activity for EST, DIAP, CAT, and PGM. Fourteen were found in EST, six in DIAP, six in PGM, three in CAT, and one in LAP (Figure 9).

Zymograms of LAP revealed monomorphism in all tested isolates, since a single anodal band was observed. Absence of bands was also observed in Gamma and alpha-Brazil European races.

PGI enzyme was monomorphic to all isolates except for CL5 for which isozyme patterns were observed. Pattern 1, composed of a single major band, was present in 17 isolates, while Pattern 2 was only observed in CL5.

Although PGI and LAP enzymes yielded well resolved patterns, little variability was detected among the C. lindemuthianum isolates. A higher level of polymorphism was observed with EST, DIAP, and PGM than with CAT among Colombian and European isolates.

Enzymes shown in Table 11 yielded sharp, well-resolved patterns, and showed electrophoretic variation among isolates of C. lindemuthianum

Table 11. Enzyme loci and isozyme patterns detected in Colombian and European races of *Colletotrichum lindemuthianum*.

Isolate	Isozyme pattern observed			
	EST[a]	DIAP	PGM	CAT
Colombia	[a]			
CL-1	5	4	7	1
CL-2	1	1	2	2
CL-3	9	2	1	2
CL-4	5	4	3	1
CL-5	8	8	6	3
CL-6	2	3	3	2
CL-7	10	1	4	2
CL-9	5	4	5	1
CL-10	1	2	2	2
Europe				
α-Brazil	11	5	4	1
C-236	7	8	1	2
Delta	3	5	1	2
Gamma	1	1	1	2
Iota	2	7	3	1
Kappa	3	8	-	2
42-80K	6	4	1	1
Lambda	1	1	1	2
Epsilon Kenya	4	6	1	2

[a] Detected enzyme loci

[b] Isozyme patterns in each detected enzyme

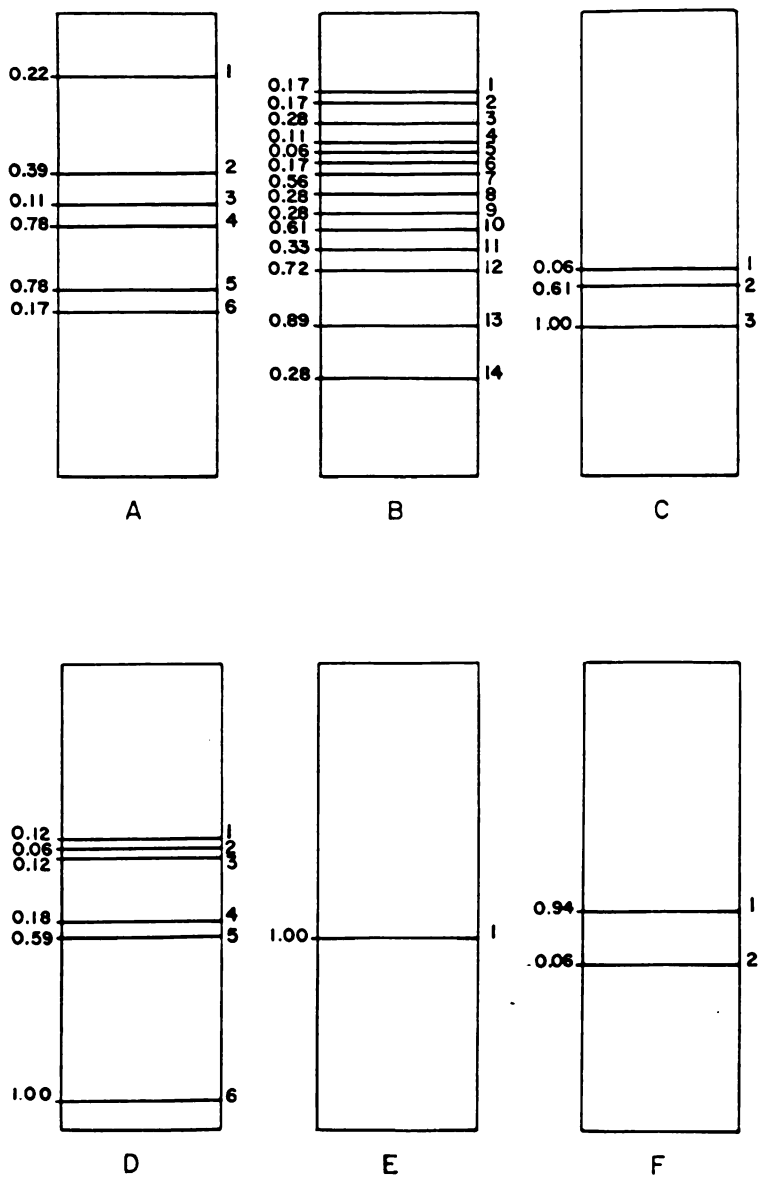

Figure 9. Total number of bands observed in each of the six enzymes; A: diaphorase; B: esterase; C: catalase; D: phosphoglucomutase; E: leucine aminopeptidase; and F: glucose phosphate isomerase. Bands num bering to the right, bands frequency to the left.

Esterase

Zymograms of EST enzyme revealed eleven different isozyme patterns (Figure 10). This enzyme showed the highest level of polymorphism among Colombian and European races of C. lindemuthianum. Patterns 2 and 5 were present in four and three isolates respectively. Patterns 1 and 2 were the only ones common to both Colombian and European races.

EST isozyme patterns revealed fourteen different bands (Figure 9B). Frequency of these bands differed among the tested isolates. Band 13, showing the highest frequency, was found in 89% of the isolates, and band 12 was observed in 72% of the isolates. The lowest frequency was observed with band 5, present in only 5% of the isolates.

Diaphorase

Eight different isozyme patterns were observed with the DIAP enzyme (Figure 11). Number of bands in the eight isozyme patterns ranged from one to four.

Zymograms of DIAP enzyme loci revealed six bands differing in frequency among isolates (Figure 9A). Bands 4 and 5 were observed in 78% of the isolates, while band 3 was present in only 11% of the isolates.

Isozyme patterns contained both Colombian and European races. Patterns 1 and 4 included four isolates, pattern 8 with three isolates, while patterns 2, 5, 3, 6, and 7 included one or two isolates.

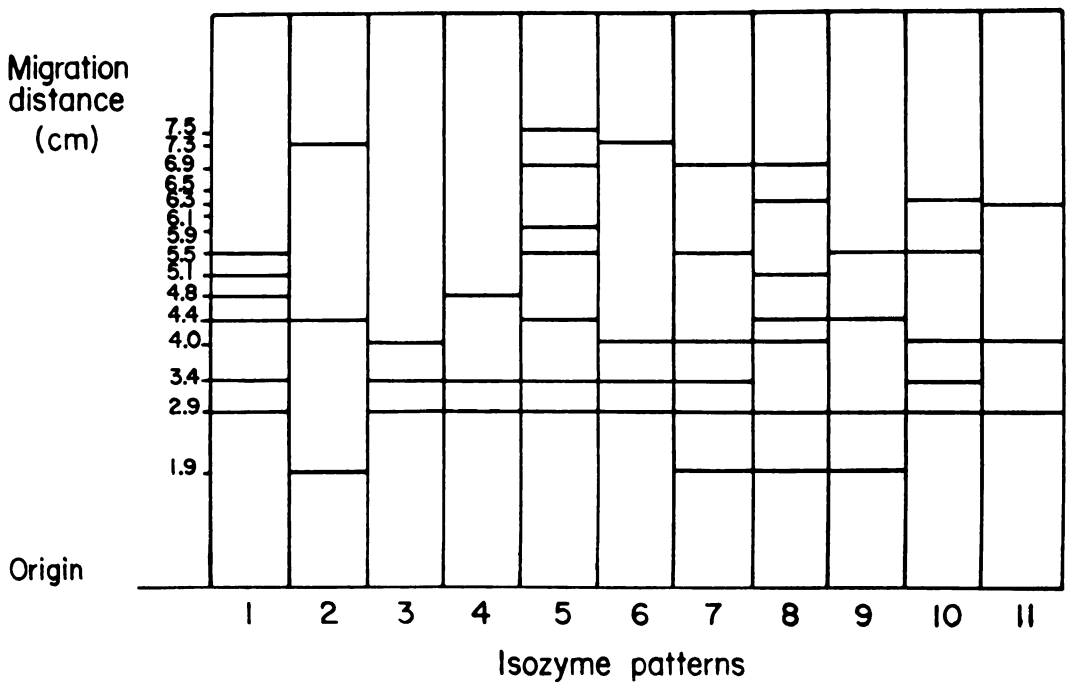

Figure 10. Esterase isozyme patterns detected in 18 isolates of Colletotrichum lindemuthianum from Colombia and Europe. Bands were resolved with buffer system I.

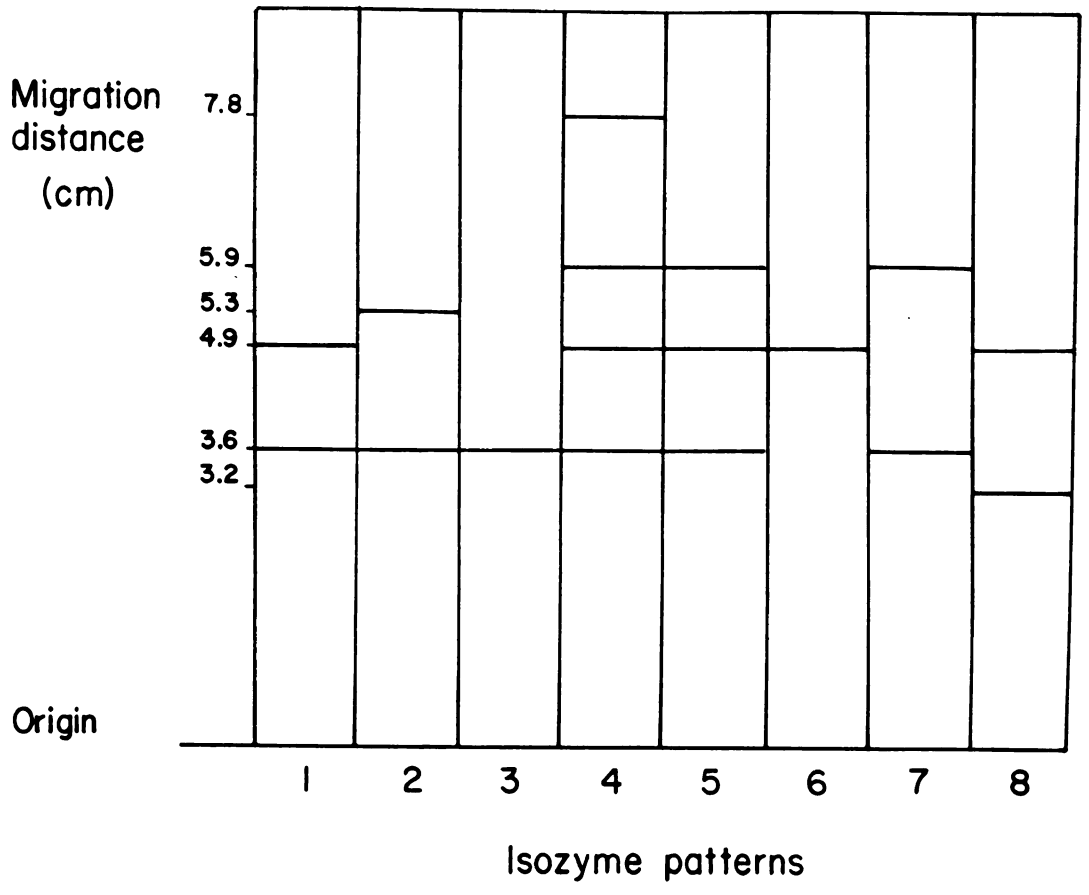

Figure 11. Diaphorase isozyme patterns observed in 18 isolates of Colletotrichum lindemuthianum from Colombia and Europe. Bands were resolved with buffer system I.

Phosphoglucomutase

Zymograms of PGM enzyme revealed seven isozyme patterns (Figure 12). A higher level of polymorphism was detected among Colombian isolates, while most European races were monomorphic to this enzyme. Six of nine European races exhibited isozyme pattern 1. Iota and Alpha-Brazil showed patterns 3 and 4 respectively, while Kappa race showed no enzymatic activity.

In contrast, isolates from Colombia exhibited seven different isozyme patterns, which indicates a high variability among Colombian population of C. lindemuthianum.

PGM isozyme patterns revealed six different bands (Figure 9D). Bands 5 and 6 were the most frequent among the isolates, with frequencies of 94% and 56% respectively.

Catalase

Analysis of the CAT enzyme loci revealed three isozyme patterns (Figure 13). Pattern 1 and pattern 2 were present in most European and some Colombian isolates while pattern 3 was present only in the CL5 isolate.

Three different bands were observed in CAT zymograms (Figure 9C). Band 3 was found in all tested isolates, while band 2 was observed in 61% of the isolates. Band 1 was present only in CL5 isolate.

Although the CAT enzyme revealed little polymorphism among C. lindemuthianum isolates, a great deal of

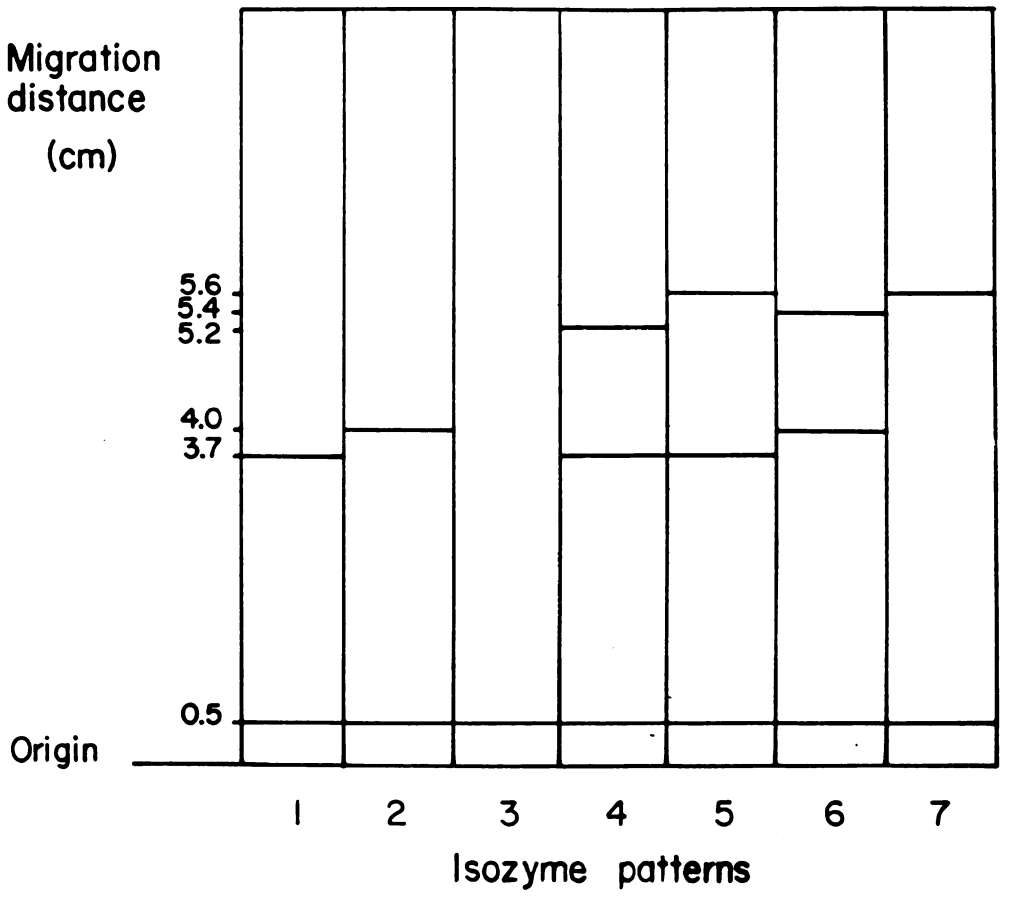

Figure 12. Phosphoglucomutase isozyme patterns observed in 18 isolates of Colletotrichum lindemuthianum from Colombia and Europe. Bands were resolved with buffer system III.

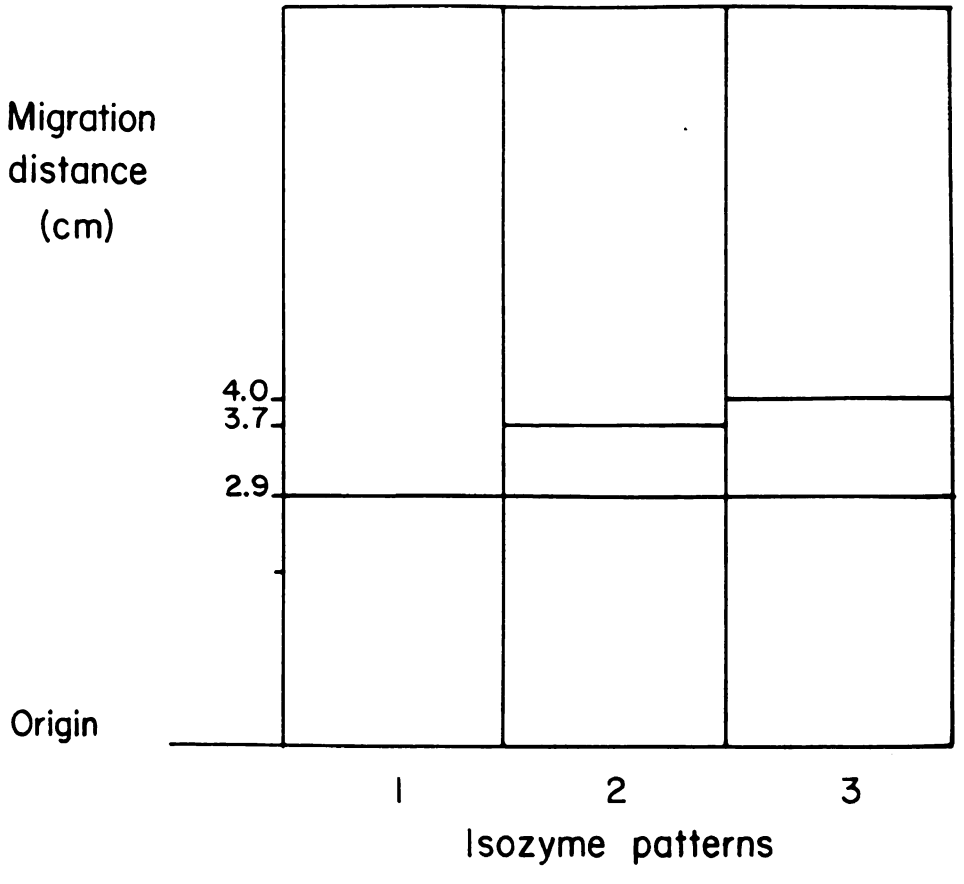

Figure 13. Catalase isozyme patterns observed in 18 isolates of Colletotrichum lindemuthianum from Colombia and Europe. Bands were resolved with buffer system I.

variability in sharpness and band numbers was observed in samples grown on different media. Composition of the media seemed to play an important role in the amount of CAT activity. CAT activity was higher in samples growing in M1 medium than in those growing in M2 and M3 media.

Host gene pool determination

Phaseolin

Phaseolin zymograms revealed a major band with three mobility variants, slow(Sanilac), fast(Tendergreen), and intermediate. Cultivars showing the intermediate variant could not be classified into a specific gene pool. Low band resolution and variation in the banding patterns were observed after repeated tests, which made phaseolin determination unreliable with the starch gel electrophoresis system.

Diaphorase

Zymograms of DIAP revealed two enzyme loci and three isozyme patterns. DIAP isozyme patterns, gene pool identity, and seed size of the twelve bean differential genotypes are compared in Table 12.

DIAP isozyme patterns have been defined as tetrameric enzyme products of seven alleles at two different loci: DIAP-1 and DIAP-2 (58). DIAP-1 contains fast, slow, intermediate, and null alleles while DIAP-2 contains fast, slow, and null alleles. Combinations of these seven alleles at DIAP-1 and DIAP-2 give rise to six different isozyme

Table 12. Seed size, diaphorase isozyme pattern, and gene pool identity of anthracnose bean differential varieties.

Differential variety	Seed size (g/100 seed)	DIAP isozyme pattern	Gene pool
AB 136	31(Medium)	S[a]	M[b]
Aguille Vert	38(Medium)	S	M
Coco a la creme	46(Large)	F	A
Cornell 49.242	28(Small)	S	M
Evolutie	22(Small)	N-2	- *
Mexico 222	30(Small)	S	M
Michelite	21(Small)	S	M
Mich.D.R.Kidney	48(Large)	F	A
Perry Marrow	54(Large)	F	A
P.I.167.399	16(Small)	S	M
P.I.165.426	18(Small)	S	M
Tendergreenc[c]	38(Medium)	F	A
Sanilac[c]	17(small)	S	M

[a] S: slow
F: fast
N-2: Null-2

[b] M: Mesoamerican gene pool
A: Andean gene pool
-: non-defined gene pool

[c] Control varieties

* S for DIAP-1 tentative classification as Mesoamerican gene pool

patterns: fast, slow, unique, null-1, null-2, and rare.

Three of these patterns were observed in the twelve bean differential cultivars: fast, slow, and null 2. The fast DIAP pattern was observed in three cultivars (Figure 14). Slow, the most predominant pattern, was found in eight cultivars, while null-2 was detected in only one cultivar.

According to Sprecher (58) materials carrying the DIAP-1 fast pattern are more likely classified into the Andean gene pool, while those carrying the DIAP-1 slow pattern are associated with the majority of small-seeded Mesoamerican lines. However, origin of the DIAP-2 null allele is not yet clear. This allele exhibits a mixture of characteristics from both Mesoamerican and Andean gene pools. It is suggested that occurrence of null-2 pattern may be associated with introgression from the Mesoamerican gene pool.

Pathogenicity tests revealed that Colombian C. lindemuthianum isolates were pathogenic on both Mesoamerican and Andean bean cultivars.

Two isolates were pathogenic only on Mesoamerican cultivars but not on Andean gene pool cultivars. Perry Marrow, Coco a la creme, and Michigan Dark Red Kidney, classified in the Andean gene pool, were resistant to inoculations with CL8 and CL9 isolates. Six Mesoamerican cultivars were susceptible to inoculation with these two isolates.Table 12. Seed size, diaphorase isozyme pattern, EST, CAT, DIAP, and PGM isozyme patterns detected in Colombian and European races of C. lindemuthianum were not

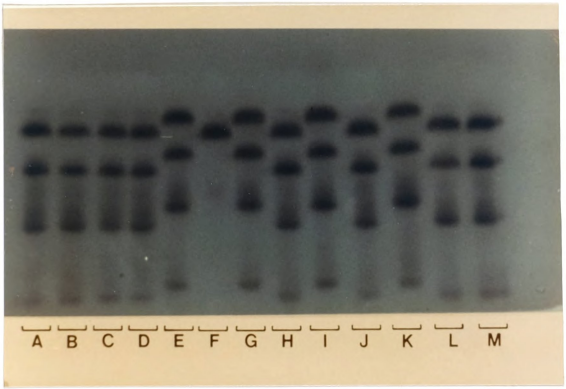

Figure 14. Diaphorase isozyme patterns detected in 12 anthracnose differential varieties. Bands were resolved with buffer system I. A: Mexico 222; B: Sanilac; C: Cornell 49242; D: Michelite; E: M.D.R.kidney; F: Evolutie; G: Coco a la Creme; H: Aguille Vert; I: Tendergreen; J: PI 165426; K:Perry Marrow; L: PI 167399; M: AB 136.

related to host gene pool nor to geographical origin of isolates. Physiological races present in C. lindemuthianum were not consistently correlated with electrophoretic enzyme patterns.

DISCUSSION

The European Delta race, Brazilian and Mexican races were detected in Colombian isolates of C. lindemuthianum. Delta group, which is common in the Brazilian races of C. lindemuthianum, was the most predominant race among the Colombian isolates. The absence of other European races among Colombian isolates agreed with results previously reported. Bokosi (11) reported a lack of similarity between Malawian isolates and European races. Also, Leakey and Simbwua (42) reported a similar finding where Ugandan isolates, appeared completely different from the European races. CIAT (26) has reported that isolates from different bean growing areas in Colombia appeared different from all reported races.

Two Colombian isolates CL8 and CL9 characterized as Alpha group were pathogenic only on small-seeded bean types. This supports the findings of Leakey and Simbwa (42) who reported that Uganda alpha and delta races were pathogenic on small-seeded bean cultivars, whereas, beta, gamma, epsilon, and zeta races were pathogenic on large-seeded bean cultivars. Melendez & Los Angeles (46) also have reported that 90% of Mexican beans were resistant to beta and gamma races.

I was unable to show any correlation between seed size and race in the present study. However, Cobo (29) indicated

that Colombian isolates of C. lindemuthianum were generally more pathogenic on large-seeded bean types. Individual mixtures of C.lindemuthianum isolates from Antioquia, Cauca, Valle, Nariño and Huila bean growing areas were inoculated on 69 Colombian commercial beans and advanced lines (29). Antioquia and Cauca mixtures were the most pathogenic among all tested mixtures. Preliminary studies on the electrophoretic variation of Colombian C. lindemuthianum isolates revealed changes in enzymatic activity as well as banding patterns.

Composition of growth media and age of the fungus seemed to alter the electrophoretic patterns. Samples grown for fourteen days on M1 medium showed the best electrophoretic characteristics. Best enzymatic activity, number of resolved bands, and detectable polymorphism among isolates of C. lindemuthianum, was observed on fourteen day-old samples grown on M1 medium. The European lambda race exhibited more variability in electrophoretic patterns due to culture conditions than the other races.

Electrophoretic analysis of the eighteen C. lindemuthianum isolates revealed these to be polymorphic to EST, CAT, PGM, and DIAP, whereas, they were monomorphic to LAP and PGI.

A degree of differentiation between Colombian and European races of C. lindemuthianum was observed only with PGM and CAT zymograms. PGM isozyme pattern 1 and CAT pattern 2 occurred in six European races.

Bands 3, 5, 6, and 13 in CAT, PGM, DIAP, and EST zymograms were found in most of the tested isolates. High incidence of these bands could be indicative of common alleles in Colombian and European races of C. lindemuthianum. Bands exhibiting low frequency among isolates may reflect new alleles, the products of mutation occurring in both populations.

Although isozyme analysis did not provide a means to identify physiological races of C. lindemuthianum, electrophoretic patterns revealed a complex genetic structure of the species, which seems to reflect the world wide diversity of races and subraces of this fungus.

Use of a standarized set of differentials for race identification in C. lindemuthianum will properly determine the world wide diversity of races and subraces of this fungus. Latin American breeders and phytopathologist have recently proposed a standarized set of differentials for the identification of Latin American races of C. lindemuthianum (M. A.,Pastor Corrales, pers. comm.). Use of this standarized set by European, African, and American researchers will provide more accurate assessment of the pathogen's variability than previously reported. Detected variability might be later analyzed by the use of molecular markers. A more extensive study, including isolates from Europe, Latin America, and Africa, might clarify the genetic variability present in populations of C. lindemuthianum.

In summary, Colombian races of C. lindemuthianum were

closely related to Brazilian and Mexican races of the fungus, where the Delta group is the most predominant.

Electrophoretic analysis of Colombian and European races of C. lindemuthianum revealed polymorphism for EST, CAT, PGM, and DIAP but monomorphism for LAP and PGI. Isozyme patterns were not related to physiological races nor to geographical origin of isolates nor to gene pool origin of the host cultivar differentials; instead they reflected a complex genetic structure of the species, complicated by culture influence such as culture age and media substrates.

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APPENDIX

APPENDIX A

Acid phosphatase (ACP)

0.1 M sodium acetate ph 5.0	25 mL
sodium α-naphthyl acid phosphate	25 mg
fast garnet GBC salt	25 mg

Adenylate kinase (AdK)

buffer solution: 1 M tris-HCL ph 8.0 buffer	1 mL
water	8 mL
α-D-glucose	100 mg
adenosine 5'-diphosphate	10 mg
hexokinase	40 units
glucose-6-phosphate dehydrogenase	10 units
NADP	5 mg
MTT	3 mg
Maldola blue	trace
agarose	70 mg

Alcohol dehydrogenase (ADH)

1 M tris-HCl pH 8.0	2.5 mL
distilled water	20 mL
95% ethanol	0.5 mL
NAD	7 mg
MTT	4 mg
PMS	trace

Catalase (CAT)

potassium ferricyanide	500 mg
ferric chloride	500 mg
distilled water	50 mL
0.01% H_2O_2	4 drops

Diaphorase (DIAP)

1 M tris-HCl pH 8.5	2.5 mL
distilled water	20 mL
NADH	7 mg
2,6 dichlorophenol indophenol	trace
MTT	10 mg

Esterase

0.1 M potassium phosphate buffer	75 mL
α-naphthyl acetate(dissolved in 10mL acetone)	150 mg
fast blue RR	75 mg

Appendix A (cont'd.)

Glucose phosphate isomerase (PGI)

1 M tris-HCl pH 8.5	2.5 mL
distilled water	20 mL
0.1 M $MgCl_2$	0.5 mL
glucose6-phosphate dehydrogenase	10 units
fructose-6phosphate	5 mg
NADP	3 mg
MTT	3 mg
Maldola blue	trace

Glutamate dehydrogenase (GDH)

1 M tris-HCL pH 7.1	2.5 mL
distilled water	20 mL
glutamate (monosodium salt)	1 g
NAD	10 mg
MTT	4 mg
maldola blue	trace

Isocitrate dehydrogenase (IDH)

1 M tris-HCL	2.5 mL
distilled water	20 mL
0.1 M $MnCl_2$	0.25mL
sodium isocitrate	15 mg
NADH	5 mg
MTT	4 mg
Maldola blue	trace

Leucine amonipeptidase (LAP)

0.1 M potassium phosphate pH 6.0	25 mL
fast black K-salt	10 mg
0.1 M $MgCL_2$	0.5 mL
L-leucyl-naphthylamide (dissolved in 1 mL of NN-dimethyl formamide)	10 mg

Malate dehydrogenase (MDH)

1 M tris-HCl pH 8.5	2.5 mL
distilled water	20 mL
L-malate	20 mg
NAD	10 mg
MTT	4 mg
PMS	trace

Appendix A(cont'd)

Malic enzyme (ME)

1 M tris-malate pH 7.2	25 mL
$MgCl_2$	2.5 mL
L-malate	10 mg
NADP	3 mg
MTT	3 mg
maldola blue	trace

Methyl umberiferyl-esterase (Mu-EST)

0.1 M potassium phosphate pH 6.0	10 mL
4-methylumbelliferyl-acetato (dissolved in 1 mL acetona)	3 mg

Peroxidase (PRX)

0.1 M sodium acetate pH 5.0	25 mL
3-amino-9-ethylcarbazole (dissolved in 3 mL NN-dimethyl formamide)	20 mg
30% H_2O_2	1 drop

Phosphoglucomutase (PGM)

1 M tris-HCl pH 8.0	2.5 mL
distilled water	20 mL
$MgCl_2$	3 mL
glucose-1-phosphate	60 mg
glucose-6-P-dehydrogenase	10 units
$MgCl_2$	0.5 mL
fructose-6-phosphate	5 mg
NADP	3 mg
MTT	3 mg
maldola blue	trace

6-Phosphogluconate dehydrogenase (6-PGDH)

0.1 M tris-malate pH 7.2	25 mL
6-phosphogluconate	6 mg
NADP	4 mg
MTT	4 mg
Maldola blue	trace

Appendix A (cont'd)

Shikimate dehydrogenase (SKDH)

1 M tris-HCL pH 8.5	2.5 mL
distilled water	22.5 mL
shikimic acid	15 mg
NADP	4 mg
MTT	3 mg
maldola blue	trace