This is to certify that the

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Pathogenic Variation, Production of Toxic Metabolites, and Isoenzyme Analysis in Phaeoisariopsis griseola (Sacc.) Ferr.

presented by

Fernando Jose Correa-Victoria

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Alfred W. Saettler
Major professor

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PATHOGENIC VARIATION, PRODUCTION OF TOXIC METABOLITES, AND ISOENZYME ANALYSIS IN *Phaeoisariopsis griseola* (Sacc.)Ferr.

By

Fernando Jose Correa-Victoria

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ABSTRACT

PATHOGENIC VARIATION, PRODUCTION OF TOXIC METABOLITES, AND ISOENZYME ANALYSIS IN *Phaeoisariopsis griseola* (SACC.)FERR.

By

Fernando Jose Correa Victoria

Angular leaf spot (ALS) of beans (*Phaseolus vulgaris* L.) caused by *Phaeoisariopsis griseola* (Sacc.)Ferr. is an important disease in several Latin American and African countries. Seventeen Latin American isolates were studied for virulence characteristics on a group of 21 bean cultivars. Large differences in virulence were found among isolates for disease severity, lesion size, lesion number, sporulation capacity, and number of days required to cause a given level of disease. No cultivar was resistant to all isolates of the pathogen; however, regression analyses indicated that several cultivars such as A 339 and BAT 1647 show presence of several components of non-specific resistance.

Forty-two isolates of the pathogen obtained from Latin America and Africa were separated into fourteen pathogenicity groups on the basis of their reactions on eight differential bean cultivars.

Studies were conducted on production of toxic metabolites *in vitro* by two *P. griseola* isolates representing the most pathogenic (Colombia 1) and the least pathogenic (Michigan 5) groups. Results indicate that isolate Colombia 1 produces several toxic compounds which differ in host-specificity on the two bean cultivars Montcalm and BAT 1647. Isolate Michigan 5 produced

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toxic metabolites with a degree of specificity towards the susceptible cultivar Montcalm. A 10 fold difference in the concentration of toxin required to induce the same symptoms on the resistant cultivar (BAT 1647) with respect to the susceptible cultivar (Montcalm) was found. Further studies are needed to elucidate the role of these toxic compounds in the ALS disease.

Isoenzyme variation among 55 P. griseola isolates from Latin America, Africa, and USA was studied. Two isoenzyme patterns were found for each of the four enzymes esterase (EST), catalase (CAT), leucine aminopeptidase (LAP), and adenylate kinase (AdK). All African isolates exhibited pattern 1 for each enzyme while Latin American isolates exhibited both pattern 1 and pattern 2. Pattern 1 of each enzyme was associated with large-seeded bean types, suggesting coevolution with this bean type in the Andean zone of South America. Pattern 2 was associated with small-seeded bean types suggesting coevolution with this bean type in Central and North America.

Dedicated to the memory of

Carmen Elena, my sister and father

Jaime Hernando, my brother and son

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I also wish to thank CIAT and specially Dr. Marcial P. Corrales for the opportunity to conduct research at the Center during the period of December of 1984 to May of 1985.

Appreciation is also extended to all the people in the laboratory that contributed in these studies.

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GENERAL INTRODUCTION

Cultivation of food legumes such as dry beans (*Phaseolus vulgaris* L.) is very extensive in numerous Latin American and African countries. The protein content of dry beans ranges between 20-25%. Hence, this grain legume is an important source of protein for a large part of the population in those areas.

Of numerous constraints to stable dry bean production around the world, diseases are among the most important. Angular leaf spot (ALS), caused by the fungus *Phaeoisariopsis griseola* (Sacc.)Ferr., is a major disease of dry beans in tropical and subtropical regions of the world. Symptoms of the disease may occur on all plant parts. On leaves, lesions are initially gray or brown and become surrounded by a chlorotic halo. Lesions become necrotic and assume the typical angular shape. Lesions may increase in size, coalesce and cause partial necrosis and yellowing of leaves followed by premature defoliation (Figure 1). Infected pods exhibit oval to circular spots with reddish brown centers surrounded by darker colored borders. Lesions on stems and branches appear as elongated brown areas and synnemata and spores are produced under favorable conditions of high humidity.

The environment most favorable for epidemic development of the disease includes moderate temperatures (16-28 C), continuous free water on foliage and stems, or high humidity for at least 48 hours, alternating with periods of low humidity. The fungus is

Figure 1. Severe symptoms of angular leaf spot (ALS) on Montcalm include necrotic lesions with typical angular shape, coalescence of lesions, and yellowing of leaves followed by premature defoliation.

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disseminated from sporulating lesions by splashing water or wind-blown soil particles and by wind currents. The fungus can overwinter or oversummer as stromatic tissue on diseased debris, and the pathogen is seed transmitted.

The disease may result in losses of up to 80 percent depending on the susceptibility of the cultivar and the environmental conditions. Recommended methods for the control of ALS include the use of fungicidal chemicals, pathogen free seed, crop rotation, and removal of infected crop debris. However, the effectiveness of these methods is limited due to the high costs of using chemical control, the ability of this pathogen to survive in plant debris for long periods and availability of land to practice crop rotation. Consequently, the development and introduction of bean cultivars resistant to ALS is regarded as an effective and economical means of reducing disease incidence and, therefore, reducing yield losses.

The Centro Internacional de Agricultura Tropical (CIAT) is responsible for developing technology, in collaboration with national programs, to increase yields in the Latin American and African countries. In order to achieve that goal, CIAT has the world's largest bean germplasm collection with over 30,000 accesssions of P. vulgaris available for research. Evaluations for reactions to important diseases are undertaken by the Bean Program at CIAT to identify sources of resistance that can be utilized for breeding purposes and for international nurseries. However, for breeding programs to succeed, the extent of

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pathogenic variation existing within the pathogen population must be determined by race surveys and virulence analysis. Disease screening, then, can be based on a widely-based range of the pathogenic spectrum in order to obtain relatively stable resistance.

Plant pathogens have long been known to produce toxic substances that play a role in pathogenesis. A better understanding of the chemical basis for plant disease resistance and susceptibility has been possible since the discovery of the role of toxic compounds as disease determinants. It is of great interest to determine if any of the toxic metabolites normally produced by pathogens in culture are associated with disease development. In many cases, these toxins have been used in plant breeding programs to screen for disease resistance.

Gel electrophoresis has been used extensively to determine the amount of isoenzyme and protein variation in populations of many organisms including fungi. The amount of inter- and intra-species variation observed for different isoenzymes in fungi has been used to separate species of several genera as well as physiological races within a specie. In addition, the study of isoenzymes in fungi has been exploited as a natural marker system for the detection of recombination _in vivo_ in different fungi in which sexual reproduction is unknown or rare.

The objectives of these investigations were:

1. To study pathogenic variation among _P. griseola_ isolates obtained from 14 different countries of Latin America and Africa;

2. To identify a set of bean cultivars that can be used to differentiate pathotypes or races of the pathogen;

3. To study the production of toxic metabolites by P. griseola and their possible role in development of disease symptoms;

4. To determine if any relationships exist between variation in isozyme patterns of the fungus, pathogenic variation, and geographical origen of pathogen isolates.

CHAPTER I

PATHOGENIC VARIATION IN *Phaeoisariopsis griseola* (Sacc.)Ferr.

LITERATURE REVIEW

Phaeoisariopsis griseola (Sacc.)Ferr. is an imperfect fungus which belongs to the order Moniliales, family Stilbaceae (4). This pathogen was first described by Saccardo in Italy in 1878 (33) who named it as *Isariopsis griseola* Sacc. Ferraris in 1909 (20) described the same fungus as *Phaeoisariopsis griseola* (Sacc.)Ferr.

The fungus produces clusters of conidiophores known as synnemata under favorable conditions of high humidity. Such structures are mainly formed on the lower surface of infected leaves after a period of 24-48 hours of 90-100% relative humidity (9). Conidia are borne on the tips of synnemata, are light or dark gray in color, cylindrical to spindleform, slightly curved, and septate (19,45).

A wide range of sizes in the reproductive structures of the fungus has been reported. The number of conidiophores in a synnemata varies, ranging from 8 to 40 (28). Variation in the length (80-500 u), and width (20-70 u) of the synnemal conidiophores has also been reported (23,24,28,34,41). Conidial dimensions have been reported as 20-80 u by 3-8 u (5,7,19,23,

24,26,28,34,46) with 1 to 7 septa(5,7,19,24,26,46).

Buruchara (7) found no correlations between morphological variations and physiological or pathogenic characteristics of pathogen isolates. A highly virulent form of P. griseola was isolated in Tanzania by Hocking (24); symptoms consisted of circular rather than angular lesions on bean trifoliolate leaves. This virulent form was morphologically indistinguishable from the typical isolates which cause angular leaf spot, and produced abundant synnemata on the leaf under surfaces as well as on the upper surface. The synnemata were longer than those reported previously for P. griseola. Hocking also obtained infection with the new form at spore concentrations as low as 10^2 spores/ml, compared to the 10^4 spores/ml necessary for infection with the common form of the pathogen.

Buruchara (7) indicated that the ALS pathogen exhibits little variation with regard to optimum growth temperature. He concluded that different isolates from diverse bean production areas did exhibit varying rates of growth and development. He also suggested that the differences in optimum growth and sporulation requirements reported in the literature may be due in part to the use of different media, different incubation conditions, or different techniques.

Buruchara (7) also found that sporulation was influenced by the isolate, temperature, and the interaction between them. The 24 C optimum temperature for sporulation he observed for most isolates is in agreement with other reports (9,35,37). However,

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Alvarez-Ayala (1) and Avila (3) found optima for sporulation to be 14-19 C and 16 C respectively.

The effects of temperature on infection and disease development have been studied by Cardona-Alvarez (9) who reported that infection and disease development may occur over a wide range of temperatures (16 to 28 C) with an optimun at 24 C. Similar results have been reported by others (25,38).

Numerous workers agree that moisture is the most critical factor necessary for epidemic development of ALS. Cardona-Alvarez (9) indicated that high humidity is very important for penetration, coremia formation and abundant sporulation while disease development and stromata formation require relatively dry atmospheres. Sindhan and Bose in 1980 (39) also indicated that precipitation and relative humidity (90-100 %) are more important than temperature for development of the disease in the field.

Little work has been done on pathogenic variability in P. griseola. Brock in 1951 (6) compared the pathogenicity of 13 isolates of the pathogen to the bean varieties Brown Beauty and Red Mexican; this work suggested the existence of pathogenic differences between the isolates. Cardona-Alvarez in 1956 (9) and Silvera in 1967 (37) working with 10 single-spore isolates and seven isolates, respectively, found no evidence of pathogenic specialization.

Marin-Villegas (27) identified 13 physiological races among a series of isolates obtained from three bean growing areas in Colombia. The isolates were purified using single spore transfer

techniques; 14 differential bean varieties were used for inoculations. However, the author did not verify the homogeneity of the host differential varieties he used, and indicated that additional studies were needed.

Alvarez-Ayala and Schwartz (2) differentiated five P. griseola isolates from Colombia and Ecuador by inoculating the bean varieties Brasil 260 (Caraota 260), Alabama 1, Red Kidney, ICA Duva, and Cauca 27. They also indicated that the isolates appeared to differ in virulence on the same variety.

In 1983, Buruchara (7) grouped 21 P. griseola isolates from different areas of Colombia and 1 isolate from Wisconsin (USA) into seven different pathotypes. He used the bean cultivars G 2575-10P-2C, Alabama No1, G 2858, ICA Duva, Caraota 260, and G 1805-1P-1C as differentials. Buruchara concluded that physiological specialization exists in P. griseola based on distinct differences in responses of the differential cultivars and to the fact that experimental conditions had little or no effect on his experiments. He proposed to designate isolates of the fungus as separate pathotypes rather than races since the genetic homogeneity of the differential cultivars was not ascertained. Conceptually, the systematic designation of races should be based on the existence of resistant genes of differential cultivars.

Nevertheless, demonstration of the existence of physiological races in fungi is usually based on either qualitative or quantitative differences arising from various

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host-pathogen interactions; reactions are thus usually categorized as either resistant or susceptible. On this basis, Correa (16) indicated that P. griseola exhibited pathogenic specialization, or the existence of races. Twelve differential cultivars were utilized to separate 30 isolates of the pathogen into five different pathogenicity groups. All twenty three isolates from Michigan and Wisconsin belonged to the same pathogenicity group while the remaining isolates, obtained from four Latin American countries and one African country, comprised the other four pathogenicity groups. Correa noted that Michigan isolates exhibited a relatively narrow host range among the isolates tested. On the other hand, isolates from Latin America exhibited the widest host range of all isolates. Less pathogenic isolates caused severe disease on large seeded-bean types.

Studies on bean varietal reactions to infection by P. griseola were first reported by Gardner and Mains (22) who found that cultivar Kentucky Wonder was the most resistant among forty common bean varieties tested. Brock in 1951 (6) grouped 164 bean lines into five groups, ranging from highly resistant to highly susceptible. The highly resistant varieties included Alabama No 1, Cafe, California Small White, Epicure, Mexico Black, McCaslan, Navy bean, Negro Costa Rica, Scotia, and Rojo Chico. Olave (31) classified the varieties Mexico 11, Mexico 12, and Cauca 27A as highly resistant.

Singh and Sharma (40) inoculated 40 bean lines during 1972 and 1973 under field conditions. Lines EC 38921, EC 44621, PLB

148, and Kentucky Wonders were completely free of disease during both years. Silvera (37) evaluated the resistance of 527 varieties of beans and found that only 2.6% were highly resistant; 46.6% moderately resistant; 28.1% susceptible; 14.8% highly susceptible; and 7.7% variable.

In another study, Santos-Filho et al (35) reported the variety Caraota 260 as highly resistant, Col. 1470 and U.I. 61 as resistant. Campos (8) reported cultivar P-524 from Colombia as highly resistant.

Some of the cultivars previously reported to be good sources of resistance to angular leaf spot in various countries have later exhibited susceptible reactions either in the same or a different country. Santos-Filho et al (35) found that the cultivars Cuva-168-N and Manteigao Preto 20, initially reported as highly resistant to P. griseola (17), were subsequently found to be highly susceptible. The authors also observed that Caraota 260 was highly resistant in Brazil. However, Alvarez-Ayala and Schwartz (2) found this bean cultivar susceptible to three Colombian isolates of the pathogen in greenhouse tests. They also found that cultivars Alabama No 1 and Cauca 27A, both previously reported as resistant, were susceptible to some of the Colombian isolates of P. griseola tested.

Schwartz et al (36) evaluated more than 13,000 accessions from the CIAT (Centro Internacional de Agricultura Tropical) germplasm bank during 1978-1981 for reactions to P. griseola. Field inoculations with a mixture of seven local isolates of the

pathogen were carried out during those years. Plants showing field resistance were then inoculated with a mixture of 15 isolates from eight different regions from Colombia at a concentration of 2-4 (10^4) spores/ml. Only 56 accessions exhibited resistant or intermediate disease reactions. The authors also observed that pathogenic variation inherent within populations of P. griseola complicates efforts to obtain sources of stable resistance by traditional screening and breeding methods. They further recommmended studies to : a) investigate pathogenic variation in P. griseola, b) develop more effective and practical systems to monitor pathogenic variability, and c) determine the nature and inheritance of new resistance sources.

In 1984, Correa (16) evaluated a group of 115 bean cultivars and breeding lines for disease reaction to a Michigan isolate of P. griseola. Susceptibility was found to be associated primarily with large seeded Cranberry and Kidney bean types. Only cultivar G 5686, a large-brown seeded type, was resistant to infection. This cultivar had been reported as resistant by Schwartz et al (36). Correa included cultivar G 5686 as a bean differential for angular leaf spot in his studies on pathogenic variation; however, he observed that one of the isolates, Michigan 31 was able to induce an intermediate disease reaction on G 5686 (16).

During the last ten years, the Bean Program at CIAT has given high priority to several bean diseases including rust, anthracnose, angular leaf spot, and common blight. Most efforts during those years were concentrated on the development and

improvement of pathogen inoculation and disease evaluation procedures for use in testing large numbers of germplasm accessions and breeding progenies.

CIAT (11) reported a dramatic difference in disease reactions in ALS disease nurseries between the 1978 and 1979 trials. Pathogenic variability was apparently expressed by the angular leaf spot pathogen when materials were inoculated with different isolates or infected naturally by isolates endemic to different locations. The bean cultivars BAT 332, BAT 76, and A 21 were highly resistant at one location during the 1978 trials. However, during 1979, BAT 332 exhibited an intermediate reaction while the other two cultivars exhibited susceptible and very susceptible reactions, respectively, at the same location. A similar situation has been observed in other countries (12). Numerous CIAT bean lines were evaluated in Brazil and Colombia for several years. Among those cultivars, A 339 was susceptible at Popayan, Colombia and resistant at Anapolis, Brazil. Cultivar A 235 exhibited an intermediate reaction at both locations.

In 1983, the Bean Program at CIAT established the Bean Angular Leaf Spot International Test (BALSIT), a nursery that includes the best sources of ALS resistance. This nursery is distributed by CIAT and planted in key areas where ALS is an important disease (13). From initial observations of selected bean lines in Popayan, Colombia and Capivara and Goiania, Brazil, cultivars A 235, BAT 76, BAT 1647, and Jalo EEP 558 were reported as resistant at all locations while cultivar BAT 332 was

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resistant in Colombia but susceptible in Brazil (13).

During 1984, CIAT distributed several disease resistance nurseries to Africa, including the BALSIT. The principal objective was to identify sources of genetic resistance to the widest spectrum of the pathogenic potential in the population of the angular leaf spot fungus (14). Another important objective was to detect the possible appearance of new races or pathogen groups that can attack previously resistant lines, and to prevent the extensive dissemination of a susceptible variety (14).

Field evaluations of the BALSIT in Latin America and Africa during 1984 strongly suggested that the angular leaf spot pathogen is highly variable (14). The bean cultivar BAT 332 was again observed to be highly resistant in Popayan, Colombia, but became severely diseased in several locations in Brazil and Argentina. Numerous of the cultivars with a resistant reaction at one or more locations in Brazil were resistant in Popayan, Colombia. Such cultivars included A 339, BAT 76, BAT 1647, and Jalo EEP 558. In addition, cultivar A 301 was highly susceptible in the locations of Capivara and Caruaru in Brazil but was resistant at Anapolis, Brazil and Popayan, Colombia.

During 1985, ALS was widespread and severe in several bean production regions of Latin America and Africa and many of the local commercial varieties were severely attacked (15). Field evaluations were conducted in Santander de Quilichao, Colombia, where the disease occurs naturally and where pathogenic variability appears to be broader than in Popayan. The results of

the BALSIT evaluations during 1985 in Mulungu, Zaire, showed that many bean lines that are resistant in Colombia and Brazil, are also resistant in Zaire and other countries of Central Africa.

Lines BAT 76 and Caraota 260 were very resistant in Zaire and Rwanda, and line A 339 resistant in Zaire (15). These lines were previously reported as resistant in more than one location of Brazil and Colombia (12,13,14). On the other hand, some lines such as Jalo EEP 558 considered as having an intermediate or resistant reaction in Brazil and Colombia, were susceptible in Zaire and Rwanda. Such field evaluations such as these strongly suggest that the P. griseola populations in Africa differ pathogenically from those in Latin America (15). Such pathogen variability indicates the desirability of evaluating resistant germplasm from Latin America in Africa; likewise, resistant lines from Africa should be evaluated in Latin America.

Preliminary results of the following studies on pathogenic variation of Phaeoisariopsis griseola isolates from Latin America have already been published (15).

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MATERIALS AND METHODS

Sources and Isolation of Isolates

Sixty isolates of Phaeoisariopsis griseola were obtained from locations around the world. Thirty-two of the isolates were from nine Latin American countries (Table 1), twenty-seven from seven African countries (Table 2), and one isolate, Michigan 5, from Alpena, Michigan (USA).

The isolates were either supplied by the Bean Pathology program at CIAT or isolated from infected bean tissue personally collected or provided by cooperating scientists.

Isolation of the pathogen and single spore cultures were accomplished using the following procedure: when lesions were present that contained synnemata with spores, a fine needle was used to remove the spores. Isolations were performed in a transfer chamber, using a stereomicroscope to observe the mass of spores. The fine needle, previously flamed, was dipped in sterile V-8 juice agar (200 ml V-8 juice, 3 g $CaCO_3$, 18 g agar, 800 ml distilled water), and then the spores were picked up with the needle avoiding any contact with the surface of the infected tissue. The spores were then transferred to the surface of fresh V-8 juice agar. Six transferences per petri dish were performed. Inoculated plates were incubated at 22-24 C, until well sporulating colonies formed six to ten days later.

Single spores isolates were then obtained from sporulating

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Guater
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Guat 4
Guat 5
Costa
CR 1
CR 2
Nicarag
Nic 1
Mexico
Mex 1
Puerto
PR 2
PR 4
Dominic
DR 1

Table 1. Isolates of *Phaeoisariopsis griseola* obtained from Latin America.

Isolate	Area where obtained
Colombia	
Col 1	Santander de Quilichao (Cauca)
Col 2	Popayan (Cauca)
Col 3	Funes (Nariño)
Col 4	Rionegro (Antioquia)
Col 5	Restrepo (Valle)
Col 6	Santander de Quilichao (Cauca)
Col 7	Paraguaicito (Quindio)
Col 8	Popayan (Cauca)
Col 9	Palmira (Valle)
Brazil	
Br 1	Sao Bento
Br 2	Sao Bento
Br 3	Caruaru
Br 4	Goiania
Br 5	Sao Bento
Br 6	(Unknown)
Br 7	Goiania
Argentina	
Arg 1	La Cocha
Arg 2	Rapelli
Arg 3	Ceibalito
Arg 4	Las Cañas
Guatemala	
Guat 1	Jutiapa
Guat 2	Monjas (Jutiapa)
Guat 3	Escuintla
Guat 4	Jutiapa
Guat 5	Cuyuta
Costa Rica	
CR 1	Esparza
CR 2	Fabio Baudrit
Nicaragua	
Nic 1	Carazo
Mexico	
Mex 1	Tepame
Puerto Rico	
PR 2	Isabella
PR 4	Isabella
Dominican Republic	
DR 1	(Unknown)

Table 2. Isolates of Phaeoisariopsis griseola obtained from Africa.

Isolate	Area where obtained
Malawi	
Mal 2	(Unkown)
Mal 3	Bunda (Lilongwe)
Mal 4	Matapwata
Mal 5	Riphondo
Mal 7	Dedza
Mal 9	Muera (Dowa)
Mal 10	Mucra (Dowa)
Mal 11	Muera (Dowa)
Tanzania	
Tz 1	Magamba
Tz 2	Milungui
Tz 3	Arusha
Tz 4	Arusha
Tz 5	Morning Site (Morogoro)
Kenya	
Ken 1	Thelca Research Station
Ken 2	Naro (Upland)
Ken 3	Kabete (Nairobi)
Uganda	
Ug 1	Kiabahinga, Kabale, Rubanda County
Ug 2	Kachwekano, Kabale, Rubanda County
Ug 3	Kamuganguzi, Kabale, Ndorwa County
Burundi	
Bu 1	Gitega
Bu 2	Gitega
Bu 3	Kamara
Rwanda	
Rw 1	Rubenheri
Rw 2	Rubenheri
Rw 3	Butawe
Zaire	
Za 1	Mulungu
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colonies in the following way: a conidial suspension was prepared from a sporulating colony in sterile-distilled water, diluted, and one or two drops from several dilutions poured onto the surface of petri dishes containing PDA (39 g of Difco PDA, 1000 ml distilled water). Inoculated plates were incubated at 22-24 C for 24 hours after which time several germinated spores were located under the stereomicroscope. A total of 12 of these spores plus a small amount of media was transfered aseptically with a fine needle to the surface of fresh V-8 juice agar. The colony showing maximum sporulation under the stereomicroscope was selected, transfered, and maintained on V-8 juice agar.

For tissue samples that did not contain sporulating lesions, infected tissue was incubated under conditions of high humidity for 48 to 96 hours. During incubation, synnemata bearing spores were produced and the same procedure as described above was followed to obtain single spore isolates.

All isolates of P. griseola were maintained on V-8 juice agar by periodic transfer of 8 to 10 highly concentrated drops of spore suspensions to fresh plates. All isolates were preserved on V-8 juice agar plates incubated at 4 C.

Inoculum Preparation and Plant Inoculation

Spores for inoculations were obtained by scraping them from plates incubated 10 to 12 days at 22-24 C. Spore concentrations were adjusted to near $2(10^{4})$/ml of distilled water containing one drop of tween 80 (Polyoxyethylene sorbitan monooleate) per 100

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Inoculum was applied as a fine mist onto upper and lower leaf surfaces of the two trifoliolate leaves on test plants 19 to 22 days old. Sprays were applied with a De Vilbiss No. 15 atomizer, attached to a compressed airline at 12 psi. and held at a distance of about 10-15 cm from the leaf.

Inoculated plants were immediately placed in a humidity chamber with relative humidity of 95-100% for four days at 22 to 28 C. Plants were removed to greenhouse benches and evaluated for their ALS reaction over the following 30 days. During the course of the experiments the greenhouse temperatures fluctuated between 19 and 30 C with a mean of 24 C.

Host Cultivars

In order to study the pathogenic variation of P. griseola, a set of 21 bean lines and varieties was initially selected (Table 3). Their selection was based on the different reactions that most cultivars have exhibited to ALS at one location, different locations within a contry, or various locations from different countries. All of the reactions have been recorded by the Bean Pathology Program at CIAT over several years, and most of this information is published in the Bean Program Annual Reports (11,12,13,14,15).

Some of the cultivars such as Alabama No. 1, G 2858, Caraota 260, G 1805, Cornell 49242, BAT 332, G 5686, Pompadour Checa, Seafearer, and Montcalm, had already been used in other studies

Table 3. Host cultivars used for differentiation of *Phaeoisariopsis griseola* isolates

Cultivar[a]	Seed characteristics		Reported ALS reaction[c]				
	Size[b]	Color	Col	Br	Arg	Za	Rw
Alabama No1 (G 4225)	medium	black	S	R			
G 2858	medium	pinto	S				
Caraota 260 (G 5698)	small	black	I-S	R		R	R
G 1805	large	cream	R	S			
A 212	small	black	R				
A 235	small	black	R-I	R-I	R		
Jalo EEP 558 (G9603)	large	cream	R-I	R		S	S
Cornell 49242(G5694)	small	black	S	R			
Bat 332	small	cream	R-I	S	S	R	R
G 5686	large	cream	I				
Pompadour Checa	medium	red mottled					
Seafarer (G 5714)	small	white					
Montcalm (G 6416)	large	red					
Calima (G 4994)	large	red mottled	I				
A 301	small	cream	I	R-S			
A 339	medium	cream	R-S	R	S	R	
BAT 1647	small	black	R	R-I	R		
A 62	medium	cream	S	S	S		
A 21	small	red	R-S				
BAT 76	small	black	R-S	R		R	R
Amendoin (G 5164)	medium	pink mottled					

[a] G is accession number (CIAT germplasm bank), A and BAT are CIAT designations.

[b] Weight of 100 seeds: small (25 g or less), medium (25-40 g), large (40 g or more)

[c] Reported ALS reaction in Colombia (Col), Brazil (Br), Argentina (Arg), Zaire (Za), and Rwanda (Rw) (11,12,13,14,15). R= resistant, I= intermediate, S= susceptible.

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as bean differentials for ALS (2,7,16). These bean cultivars were logically included to make possible comparisons with previous reports of pathogen races.

Seeds of most cultivars were obtained from the Bean Germplasm Bank Collection and the Bean Pathology Program of CIAT. Seeds of Montcalm, Seafarer, and Pompadour Checa were obtained from Dr. A.W. Saettler and Dr. J.D. Kelly at Michigan State University. Single plant selection and seed increase were performed for all of the bean cultivars prior to these studies.

Seed size and color were two characteristics considered in the selection of the host cultivars since Correa (16) observed that disease resistance to Michigan isolates of the pathogen was associated mainly with navy, black, and pinto bean types, while susceptibility was found in red kidney and cranberry types.

Evaluation Scale

Disease severity assessment was based on percentages of leaf affected by P. griseola according to a CIAT evaluation scale of 1 to 9 (Figure 2) where:

1- no visible symptoms of the disease or presence of small lesions on leaves affecting up to 1% of the tissue area

3- Approximately 5% of the tissue area affected by the lesions. Presence of small lesions with low or no sporulation

5- Approximately 10% of the tissue area affected by the lesions

7- Approximately 25% of the tissue area affected by the lesions

9- Approximately 50% or more of the tissue area affected by

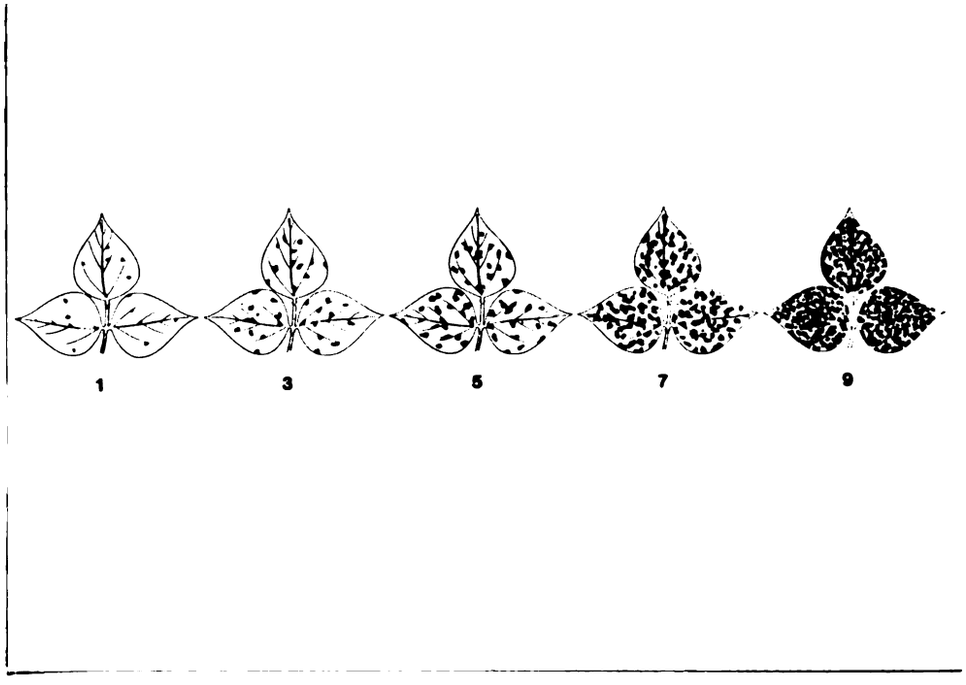

Figure 2. Scale used for evaluating disease reactions caused by P. griseola:
1= no symptoms, or less than 1% leaf area covered by ALS lesions.
3= approximately 5% of leaf area covered by lesions
5= approximately 10% of leaf area covered by lesions
7= approximately 25% of leaf area covered by lesions
9= approximately 50% of leaf area covered by lesions

lesions causing severe defoliation

Plants were evaluated every other day after symptoms were initially seen.

Studies on Pathogenicity and Virulence Characteristics of 17 Phaeoisariopsis griseola isolates from Latin America

These studies were conducted at the Centro Internacional de Agricultura Tropical (CIAT), Cali, Colombia during the period of December 1984 to May 1985. A series of 21 bean cultivars (Table 3) were inoculated with 17 isolates of P. griseola from seven different Latin American countries. Seeds were planted in 15 cm diameter pots containing a steam sterilized mixture of soil and sand (5:1 V/V). Two pots per cultivar with three plants per pot were manitained at 20 to 24 C in a greenhouse bench until the time of inoculation. Inoculation and incubation of plants were performed as previously described. The experiments were repeated two times.

Disease Severity, Incubation Period, and Lesion Number

Disease reactions were evaluated beginning 8 days after inoculation and continuing for at least 30 days or until defoliation occurred. Disease severity, incubation period, and lesion number were determined using the same set of experimental materials.

Disease severity on each cultivar was based on the evaluation scale described in Figure 2, and is expressed as

percentage of infected leaf area. A total of six plants were evaluated each time and the mean calculated.

Incubation period was expressed as the time required for an isolate to cause 20% of disease severity on a particular cultivar.

Numbers of lesions were counted every other day after symptoms first appeared until the number was constant or until lesions coalesced.

Lesion Size

Lesions were measured using a linear metric ruler and expressed in square millimeters. A total of 3 lesions per plant on six plants was measured at random every two days after symptoms appeared until the lesion size was constant. Lesion size was expressed as the average of the 18 measurements.

Sporulation Capacity

Two plants per cultivar were incubated at 95-100% relative humidity for 48 hours after angular leaf spot lesions on each plant were well formed. Spore formation has been reported to be maximum after exposure of lesions for a period of 24-48 hours under high humidity conditions (9).

After 48 hours, plants were allowed to dry, and infected leaves were collected and taken to the laboratory. Five lesions per leaflet were measured with a linear metric ruler at random, excised with a razor blade under a stereomicroscope and placed in

1 ml of water in a test tube. Two replications per treatment were performed. Test tubes containing the samples were then vigorously shaken for two minutes. From each spore suspension, four counts were made by means of a hemacytometer (American Optical Corporation, Buffalo, New York). The sporulation capacity was then expressed as the number of spores produced per square millimeter of lesion.

Pathogenicity Groups in 42 Phaeoisariopsis griseola Isolates from Latin America and Africa Using a Differential Set of eight Bean Cultivars

These studies were conducted at Michigan State University, during the period of June 1985 to May 1986. A set of eight bean cultivars was inoculated with Latin American and African isolates of P. griseola. Seeds were planted in 15 cm diameter pots containing a mixture of steam sterilized soil and vermiculite (2:1 V/V). Three pots per cultivar with two plants per pot were maintained in a greenhouse bench until the time of inoculation. Inoculation technique and incubation of plants after inoculation were performed as previously described.

Disease severity and incubation period were determined as described previously. The pathogenicity groups were arranged according to an arbitrary classification of disease reaction by each one of the bean cultivars. The criteria used were as follows:

S: Susceptible, more than 5% leaf area covered by P.

griseola lesions

I: Intermediate, 1-4% leaf area covered by lesions

R: Resistant, no disease symptoms or less than 1% leaf area covered by lesions

RESULTS

Studies on Pathogenicity and Virulence Characteristics of 17 Phaeoisariopsis griseola Isolates from Latin America on 21 Host Cultivars

The results clearly reveal wide variability of P. griseola in terms of pathogenicity and virulence of isolates.Pathogenicity was based on the number of cultivars on which an isolate caused 1% or more infection. Pathogenicity differed among isolates from different countries as well as among isolates from within the same country. The most pathogenic isolates caused disease on all 21 bean cultivars tested while the least pathogenic isolates caused disease on only 4 cultivars of the 21 (Table 4). Cultivars tested are described in Table 3.

Of the 17 isolates, two were pathogenic to all 21 cultivars; two to 19; three to 17; two to 14; one to 13; one to 11; two to 5; and four to 4 cultivars (Table 4). The two most pathogenic isolates were those from Santander de Quilichao, Colombia (Col 1), and from Jutiapa, Guatemala (Guat 1). Both isolates were pathogenic on all 21 cultivars tested.

There was a smaller group of isolates exhibiting narrower host ranges, which included Colombia 3 and Colombia 4, each infecting 5 cultivars, and isolates Nicaragua 1, Argentina 2, Argentina 3, and Argentina 4, all of which infected only four cultivars. In all cases where different isolates infected the

Table 4. Pathogenicity and disease severity differences among 17 *Phaeoisariopsis griseola* isolates from Latin America based on their reactions on 21 bean cultivars.

Isolate	Area where obtained	No. infected cultivars[b]	Disease severity[a] Ave (%)[c]	(range)[d]
Colombia 1	Sant. de Quilichao	21	40	10-50
Guatemala 1	Jutiapa	21	26	7-40
Mexico 1	Tepame	19	27	2-50
Guatemala 2	Monjas	19	16	2-40
Argentina 1	La Cocha	17	22	2-50
Brazil 1	Sao Bento	17	24	3-43
Brazil 3	Caruaru	17	22	2-50
Costa Rica 1	Esparza	14	21	3-40
Colombia 2	Popayan	14	11	2-40
Brazil 2	Sao Bento	13	10	2-40
Brazil 4	Goiania	11	13	3-39
Colombia 3	Funes	5	40	27-50
Colombia 4	Rionegro	5	45	38-50
Nicaragua 1	Carazo	4	34	25-40
Argentina 2	Rapelli	4	25	4-40
Argentina 3	Ceibalito	4	32	8-40
Argentina 4	Las Cañas	4	41	35-50

[a]Percentage of leaf area covered by lesions. Minimum 0%, Maximum 50%.

[b]Plants of twenty-one bean cultivars were inoculated with each isolate by spraying with a spore suspension containing 2×10^4 spores/ml.

[c]Average disease severity over the number of infected cultivars. Six plants per cultivar were inoculated.

[d]Minimum and maximum disease severity average induced by each pathogen isolate.

same number of bean cultivars, the cultivars were also the same. This indicates that isolates infecting the same cultivars probably belong in a common pathogenicity group. However, as will be mentioned later, isolates differed greatly in amount of disease induced on a given cultivar.

P. griseola isolates from the same country differed in their pathogenicity patterns. For example, of four isolates from Colombia, isolate Colombia 1 from Santander de Quilichao infected all 21 bean cultivars while isolate Colombia 2 from Popayan infected 14 cultivars. Isolates Colombia 3 from Funes, Nariño, and Colombia 4 from Rionegro, Antioquia, only infected the same 5 cultivars. A similar situation involved the four pathogen isolates from Argentina. Isolate Argentina 1 from La Cocha was very pathogenic while the other three isolates from Rapelli, Ceibalito, and las Cañas locations were less pathogenic.

On the other hand, four isolates from three different locations of Brazil each infected a large number of the bean cultivars. Isolates Brazil 3, and Brazil 1 from Caruaru and Sao Bento respectively both infected 17 bean cultivars. Isolates Brazil 2 (Sao Bento) and Brazil 4 (Goiania), infected 13 and 11 cultivars respectively.

The four remaining isolates of the pathogen namely Mexico 1, Guatemala 2, Costa Rica 1, and Nicaragua 1 were all from Central America and all exhibited a different range of pathogenicity. Nicaragua 1, was less pathogenic, and infected only four cultivars while isolates Mexico 1 and Guatemala 2 both infected

19 cultivars and Costa Rica 1 infected 14 cultivars.

Virulence was estimated by comparing the percentage of disease caused by isolates on the infected cultivars. Table 4 shows those values for each isolate. The mean was taken over the number of cultivars infected by each isolate. It should be noted that according to the evaluation scale, the maximum infection rating given to a any particular host-isolate combination is 50%.

Variation in virulence among isolates was observed in terms of average disease severity. There was no correlation between pathogenicity and virulence.

Virulence can also be estimated by the number of lesions induced by a particular P. griseola isolate on the host plant. Average number of lesions associated with each of the 17 P. griseola isolates is shown in Table 5. Also shown is the number of cultivars infected by each isolate as well as the range in the number of lesions produced on the infected cultivar.

The lowest average number of lesions was 19 for isolate Brazil 2 while the highest average was 260 for isolate Nicaragua 1. Variation in number of lesions was observed among isolates of different countries, isolates from the same country, isolates which were highly pathogenic, and isolates which were less pathogenic.

P. griseola isolate Mexico 1 induced the highest average number of lesions (159) among isolates in the high pathogenicity group while isolate Nicaragua 1 induced the highest (260) number of lesions among isolates in the low pathogenicity group. The

Table 5. Numbers of lesions caused by 17 Phaeoisariopsis griseola isolates from Latin America on 21 bean cultivars.

Isolate	Infected Cultivars[a]	Number of lesions Average[b]	(range)[c]
Colombia 1	21	121	57-286
Guatemala 1	21	66	19-162
Mexico 1	19	159	32-464
Guatemala 2	19	30	13-100
Argentina 1	17	89	11-243
Brazil 1	17	89	26-217
Brazil 3	17	82	6-209
Costa Rica 1	14	92	15-246
Colombia 2	14	24	2- 83
Brazil 2	13	19	2- 49
Brazil 4	11	65	15-132
Colombia 3	5	172	78-312
Colombia 4	5	181	108-252
Nicaragua 1	4	260	159-429
Argentina 2	4	60	19-107
Argentina 3	4	124	48-193
Argentina 4	4	149	100-197

[a]Plants of twenty-one bean cultivars were inoculated with each pathogen isolate by spraying with a spore suspension containing $2x10^4$ spores/ml.

[b]Average number of lesions over the number of infected cultivars induced by each isolate.

[c]Minimum and maximum number of lesions induced by each pathogen isolate.

range of numbers of lesions caused by each isolate on the different cultivars indicates that isolate Mexico 1 caused the largest number of lesions (464 in at least one cultivar) while isolate Colombia 2 caused the lowest number (83 lesions). Interestingly, the lowest number of lesions was caused by isolates in the high pathogenicity group. Isolates Colombia 2 and Brazil 2 caused only 2 lesions in at least one bean cultivar. However, isolates of low pathogenicity did not infect those cultivars exhibiting low number of lesions to the more pathogenic isolates. The four bean cultivars infected by the less pathogenic isolates however, were infected by all isolates with a higher degree of pathogenicity.

The data indicate a high positive correlation, (r: 0.79 significant at P: 0.01), between disease severity and number of lesions caused by the 17 P. griseola isolates studied on all cultivars.

Another component of virulence relates to size of lesion. Table 6 shows that the average lesion size produced by P. griseola isolates varied greatly. The largest average lesion size (20.9 mm^2) among infected cultivars was produced by isolate Argentina 2. The smallest average lesion size (4.4 mm^2) was caused by isolate Brazil 3.

The range of lesion size for each isolate indicates that this character is highly dependent on host cultivar. For example, isolate Brazil 2 produced lesions of 1 mm^2 on at least one bean cultivar while the same isolate produced lesions 40 mm^2 on at

Table 6. Size of lesions caused by 17 Phaeoisariopsis griseola isolates from Latin America on 21 bean cultivars.

Isolate	Infected Cultivars[a]	Lesion size	
		Average (mm^2)[b]	(range)[c]
Colombia 1	21	11.9	11-38
Guatemala 1	21	15.4	5-34
Mexico 1	19	10.7	1-25
Guatemala 2	19	16.9	5-45
Argentina 1	17	5.4	1-16
Brazil 1	17	5.3	1-15
Brazil 3	17	4.4	1-14
Costa Rica 1	14	7.5	1-28
Colombia 2	14	12.2	1-36
Brazil 2	13	11.9	1-40
Brazil 4	11	5.7	1-16
Colombia 3	5	9.0	4-21
Colombia 4	5	6.0	3- 8
Nicaragua 1	4	4.8	2- 7
Argentina 2	4	20.8	5-34
Argentina 3	4	6.5	2- 8
Argentina 4	4	6.0	4- 7

[a]Plants of twenty-one bean cultivars were inoculated with each of 17 pathogen isolates by spraying with a spore suspension containing $2x10^4$ spores/ml.

[b]Average lesion size induced by each isolate over the number of infected cultivars. Three lesions on each of six plants per cultivar were measured until lesions reached a maximum size.

[c]Minimum and maximum lesion size induced by each pathogen isolate.

least one other cultivar. This character also seems to be independent of the level of pathogenicity. A slightly pathogenic isolate such as Nicaragua 1 caused average lesion size of 4.8 mm^2 while another slightly pathogenic isolate, Argentina 2, caused the largest lesion size average of 20.8 mm^2.

There was no significant correlation between disease severity and lesion size (r: 0.25, P: 0.05). A low negative correlation (r:-0.49, P: 0.05) existed between average number of lesions and average of lesion size.

Another aspect of virulence was the aggressiveness of P. griseola isolates, which was defined as the number of days after inoculation for the 20% level of disease severity to develop. The most aggressive isolates required an average of as few as 13 to 14 days to cause 20% disease severity while less aggressive isolates required 19 to 20 days (Table 7).

None of the most pathogenic isolates caused more than 20% of disease severity on all of the susceptible cultivars (Table 7). The most virulent isolate was Colombia 1 which caused more than 20% of disease severity on 19 of the 21 cultivars. The same isolate was also very aggressive, requiring an average of 14 days after inoculation to produce at least 20% disease severity.

Most of the less pathogenic isolates, which include those isolates infecting 4 or 5 out of 21 bean cultivars, caused more than 20% of disease severity on most of the cultivars (Table 7). These isolates however, varied in their aggressiveness to produce that amount of disease. The more aggressive ones required only 13

Table 7. Aggressiveness differences among 17 Phaeoisariopsis griseola isolates from Latin America based on their reactions on 21 bean cultivars.

Isolate	Infected cultivars[a]	Number of days after inoculation to 20% disease severity		Number of cultivars exhibiting 20% or more disease
		Average[b]	(range)[c]	
Colombia 1	21	14	10-22	19
Guatemala 1	18	18	13-22	15
Mexico 1	19	13	11-15	13
Guatemala 2	19	19	17-22	8
Argentina 1	17	15	13-20	10
Brazil 1	17	14	11-20	9
Brazil 3	17	16	12-23	10
Costa Rica 1	14	18	15-20	9
Colombia 2	14	19	17-20	5
Brazil 2	13	20	19-23	4
Brazil 4	11	16	13-19	6
Colombia 3	5	14	12-20	5
Colombia 4	5	15	13-20	5
Nicaragua 1	4	15	14-16	4
Argentina 2	4	17	17-17	3
Argentina 3	4	13	11-15	3
Argentina 4	4	14	13-16	4

[a]Plants of twenty-one bean cultivars were inoculated with each pathogen isolate by spraying with a spore suspension containing $2x10^4$ spores/ml.

[b]Average number of days after inoculation to 20% disease severity. Average is expressed over number of cultivars exhibiting 20% disease or more.

[c]Minimum and maximum number of days after inoculation to induce 20% disease severity by each pathogen isolate.

days while the less aggressive ones required 17 days.

Aggressiveness of an isolate was independent of the number of cultivars infected. Isolate Argentina 3, which infected only 4 cultivars, was as aggressive as isolate Mexico 1 which infected 19 cultivars. On the other hand, isolate Guatemala 2 which infected 19 cultivars required as many as 19 days to attain 20% disease, while isolate Argentina 2, which infected only 4 cultivars, required 17 days (Table 7).

Ten was the lowest number of days required for 20% disease production. Colombia 1 was the only isolate that exhibited this degree of aggressiveness. At the other extreme, several isolates required at least 23 days to produce the same amount of disease.

Variation in aggressiveness was also observed among isolates within a country as shown in Table 7 for isolates from Colombia, Brazil, and Argentina.

A significant negative correlation (r:-0.71, P:0.01) was found between disease severity and number of days to 20% infection.

Another factor involved with virulence is the production of secondary inoculum, or sporulation. Determination of spore production in lesions on an infected plant is difficult under laboratory conditions. There are few standard methods available to study sporulation and its relationship to virulence.

Table 8 summarizes data on number of spores per mm^2 produced by nine pathogen isolates. Premature defoliation due to severe disease development did not allow the study of spore

Table 8. Number of spores produced by nine isolates of *Phaeoisriopsis griseola* from Latin America.

Isolate	No. of spores per mm²		No. of cultivars studied[c]	Infected cultivars[d]
	Average[a]	(range)[b]		
Argentina 3	343	275-463	4	4
Colombia 2	296	139-800	10	14
Brazil 1	256	23-508	16	17
Colombia 3	190	83-312	5	5
Brazil 3	148	48-230	6	17
Argentina 1	144	33-476	15	17
Brasil 2	139	37-286	10	13
Colombia 1	129	5-377	20	21
Colombia 4	110	56-159	5	5

[a]Average number of spores produced per mm² by each pathogen isolate over thenumber of cultivars studied.

[b]Minimum and maximum number of spores/mm² produced by each isolate.

[c]Two infected plants per cultivar were incubated at 100% relative humidity for 48 hours to induce sporulation. Five lesions per plant were measured, excised and placed in 1 ml of water in a test tube. Spore concentration in each tube was calculated with a hemacytometer and expressed as spores per square millimeter.

[d]Total number of cultivars infected out of 21 inoculated.

production on all of the bean cultivars infected by a particular pathogen isolate.

The results reveal large variation among isolates in ability to produce spores within a lesion. Sporulation ranged from a low of 5 to a high of 800 spores/mm^2. Sporulation appeared independent of pathogenicity (Table 8). Isolate Colombia 1, pathogenic on 21 cultivars, exhibited the same average sporulation as isolate Colombia 4 which was pathogenic on only 5 bean cultivars. Isolates Argentina 3 and Colombia 2 ranked highest in terms of sporulation, yet exhibited different degrees of pathogenicity.

Since spore production per unit area of host tissue is an important factor from an epidemiological standpoint, correlation coefficients of this character with other factors of pathogenicity and virulence were determined.

The results indicate very low, and nonsignificant correlations between spore production and the following characters: disease severity, (r: -0.25); number of lesions, (r: -0.20); lesion size, (r: 0.01); incubation period, (r: -0.13).

Disease Reaction of 21 Bean Cultivars to *Phaeoisariopsis griseola*

Each of 21 cultivars was inoculated separately with the 17 isolates of *P. griseola* from different Latin American countries (Table 4). Disease reactions were expressed in terms of disease severity, number of lesions, lesion size, and incubation period.

The general results showed that no cultivar was resistant to

Table 9. Disease severity in 21 bean cultivars inoculated with 17 isolates of Phaeoisariopsis griseola.

Cultivar	Disease severity[a] Average[b] %	Range[c]	Regression coefficient (b)[d]	Standard error (se)	Deviation from regression
A 339	3	0-43	0.88 *	0.20	802
A 212	5	0-43	0.88 *	0.17	617
BAT 76	6	0-43	1.03 *	0.17	566
BAT 1647	7	0-50	1.20 *	0.25	1,249
A 235	7	0-50	1.11 *	0.23	1,029
Caraota 260	7	0-43	0.87 *	0.31	1,910
Cornell 49242	9	0-48	1.10 *	0.38	2,858
A 21	11	0-50	1.64 *	0.18	695
Pompadour Checa	13	0-50	1.52 *	0.38	2,898
G 1805	14	0-50	1.34 *	0.35	2,459
G 5686	16	0-47	0.85 *	0.41	3,488
A 62	17	0-40	1.44 *	0.29	1,699
G 2858	18	0-50	1.46 *	0.28	1,613
Amendoin	18	2-50	0.12 ns	0.38	3,032
A 301	19	0-50	1.40 *	0.43	3,733
Seafarer	20	0-50	1.16 *	0.42	3,580
Alabama No 1	20	0-50	1.36 *	0.43	3,741
BAT 332	22	0-50	1.87 *	0.38	2,927
Calima	26	2-50	0.19 ns	0.41	3,495
Jalo EEP 558	28	4-50	0.17 ns	0.42	3,517
Montcalm	34	6-50	0.04 ns	0.42	3,611

[a]Percentage of leaf area covered by ALS lesions after inoculation with each of 17 P. griseola isolates. Minimum and maximum disease ratings were 0% and 50% respectively.
[b]Average disease severity exhibited by each cultivar. Six plants per cultivar were inoculated by spraying plants with a spore suspension containing $2x10^4$ spores/ml
[c]Minimum and maximum disease severity exhibited by each cultivar.
[d]Regression of disease severity/cultivar/isolate on the stability index calculated over all cultivars and all isolates (10).
*: significantly different from zero (P: 0.01).
ns: not significantly different from zero.

all isolates of the pathogen, while several cultivars were susceptible to all isolates.

Lower disease severities developed in cultivars A 339, A 212, Bat 76, BAT 1647, A 235, and Caraota 260 (Table 9). In order to determine the stability of non-specific resistance in these six bean cultivars to all 17 P. griseola isolates, regression analysis indicated that they were not highly resistant to all isolates. The slope of the regression lines were significantly different from zero for all six cultivars (Table 9). This is evident when one considers the range of disease severity for the six cultivars (Table 9), yet the same cultivars also had the smallest deviations from regression.

On the other hand, cultivars Amendoin, Calima, Jalo EEP 558, and Montcalm had large average disease severities and large deviations from regression, although the regression coefficients did not deviate significantly from zero. Many bean cultivars were in between the two extremes of disease severity, had regression coefficients different from zero, and had large deviations from regression.

There was a large variation in resistance of bean cultivars as defined in terms of lesion number (Table 10).

Fewest numbers of lesions were induced on cultivars A 339 (10 lesions) and Cornell 49242 (12 lesions), while the largest numbers were induced on cultivars Montcalm and Jalo EEP 558. Cultivars A 339 and Cornell 49242 had regression coefficients not significantly different from zero and had small deviations from

Table 10. Number of lesions in 21 bean cultivars inoculated with 17 isolates of Phaeoisariopsis griseola.

Cultivar	No. of Lesions[a] Average[b]	Range[c]	Regression coefficient (b)[d]	Standard error (se)	Deviation from regression
A 339	10	0-153	0.43 ns	0.24	18,001
Cornell 49242	12	0- 73	0.32 ns	0.14	6,645
BAT 76	23	0-118	0.64 *	0.16	8,075
A 235	25	0-110	0.81 *	0.14	6,348
A 212	27	0-141	0.98 *	0.15	7,190
G 2858	27	0- 87	0.65 *	0.12	4,610
BAT 1647	28	0-187	0.78 *	0.33	34,523
Caraota 20	29	0-224	0.54 ns	0.39	47,095
A 21	36	0-172	1.17 *	1.79	10,174
Seafarer	41	0-188	0.73 *	0.30	29,192
G 1805	46	0-243	0.91 *	0.41	53,681
A 301	48	0-137	1.05 *	0.28	24,943
A 62	51	0-188	1.24 *	0.27	23,640
Pompadour Chec	58	0-286	1.91 *	0.47	68,673
G 5686	60	0-221	1.37 *	0.32	32,642
Alabama NO. 1	61	0-209	1.25 *	0.38	47,044
BAT 332	69	0-216	1.68 *	0.33	33,799
Amendoin	84	8-331	1.30 *	0.47	71,569
Calima	98	4-282	1.15 *	0.48	73,510
Montcalm	139	20-296	0.93 ns	0.56	98,053
Jalo EEP 558	162	32-464	1.16 ns	0.88	247,903

[a]Number of lesions exhibited after inoculation with each of 17 P. griseola isolates.

[b]Average number of lesion exhibited by each cultivar. Six plants per cultivar were inoculated with a spore suspension containing $2x10^4$ spores/ml.

[c]Minimum and maximum number of lesions exhibited by a particular cultivar.

[d]Regression of number of lesions/cultivar/isolates on the stability index calculated over all cultivars and all isolates (10).

*: Significantly different from zero (P= 0.01)

ns: Not significantly different from zero

regression. This suggests a high stability of these two cultivars for low number of lesions when inoculated with all 17 isolates of the pathogen. On the other hand, Montcalm and Jalo EEP 558 had larger numbers of lesions and greater deviations from regressions, although the regression coefficients did not deviate significantly from zero (Table 10).

A significant positive correlation (r: 0.86, P: 0.01) was found between average disease severity and average number of lesions on the 21 bean cultivars inoculated with the 17 P. griseola isolates.

Cultivars varied relative to size of lesions (Table 11), as they did to disease severity and number of lesions.

Cultivars A 339 and BAT 1647 developed lesions of the smallest average size, 0.9 and 1.7 mm^2 respectively, when compared to average lesion sizes on G 2858 (13.2 mm^2) and Seafarer (15.0 mm^2). Regression coefficients for the latter two cultivars were not significantly different from zero and deviations from regression were small (Table 11).

Some other cultivars had low average lesion size but either had regression coefficients different from zero or large deviations from regression, indicating less stability for lesion size. Interestingly, cultivar Amendoin had a low average lesion size, regression coefficient not significant from zero, and a small deviation from regression. This cultivar did not show the same stability for the two factors studied above, disease severity and number of lesions.

Table 11. Lesion size on 21 bean cultivars inoculated with 17 isolates of Phaeoisariopsis griseola.

Cultivar	Lesion size[a] Average[b] (mm^2)	Lesion size[a] range[c] (mm^2)	Regression coefficient (b)[d]	Standard error (se)	Deviation from regression
A 339	0.9	0-11	0.27 ns	0.13	101
BAT 1647	1.7	0-16	0.36 ns	0.19	220
BAT 76	3.5	0-15	0.82 *	0.12	96
A 235	3.6	0-20	0.95 *	0.17	186
A 212	3.8	0-20	0.95 *	0.17	185
Caraota 260	4.1	0-15	0.89 *	0.14	122
G 5686	4.2	0-21	0.09 ns	0.27	443
A 21	4.4	0-19	1.16 *	0.10	63
Amendoin	4.6	1-10	0.18 ns	0.16	84
Pompadour Checa	4.7	0-28	0.79 *	0.35	778
Cornell 49242	5.1	0-36	1.50 *	0.45	1,284
Jalo EEP 558	6.7	2-16	0.36 *	0.16	155
Alabama No. 1	6.8	0-23	1.07 *	0.24	360
A 62	7.2	0-19	1.00 *	0.23	332
G 1805	7.6	0-25	1.37 *	0.28	491
Calima	7.9	2-33	0.02 ns	0.38	906
A 301	8.6	0-31	1.72 *	0.31	601
BAT 332	8.8	0-32	0.20 *	0.28	490
Montcalm	8.8	2-34	0.25 ns	0.39	949
G 2858	13.2	0-38	0.27 *	0.29	504
Seafarer	15.0	0-45	2.52 *	0.36	798

[a]Lesion size in mm^2 after inoculation with each of 17 P. griseola isolates.

[b]Average lesion size exhibited by each cultivar. Six plants per cultivar were inoculated with each of 17 isolates of the pathogen by spraying plants with a spore suspension containing $2x10^4$ spores/ml. Three lesions per plant were measured with a metric ruler.

[c]Minimum and maximum lesion size exhibited by a particular infected cultivar.

[d]Regression of lesion size/cultivar/isolate on the stability index calculated over all cultivars and isolate (10).
*: Significantly different from zero (P= 0.01).
ns: Not significantly different from zero.

A significant positive correlation (r: 0.65, P: 0.01) was found between disease severity and lesion size for the 21 bean cultivars. On the other hand, no significant correlation (r: 0.25, P: 0.05) was found between lesion number and lesion size.

Resistance to P. griseola was also measured in terms of number of days to development of 20% disease severity (incubation period). Table 12 shows the incubation period, range, and number of isolates inducing 20% or more severity on 21 bean cultivars.

Incubation period may be inversely related to host resistance. The best information on a cultivar should be obtained from analyzing the three sets of data given in Table 12 since each cultivar exhibited 20% or more disease severity to a different number of isolates.

The results indicate that the longest incubation period, 23 days, was expressed in cultivars G 5686 and BAT 1647. The shortest incubation period was 10 days which was expressed in several cultivars, including BAT 1647. These results indicate that the incubation period for each cultivar depended upon the pathogen isolate used. In general, cultivar G 5686 had the longest average incubation period (19 days). Seven isolates caused more than 20% of disease severity on this cultivar with a range of 14 to 23 days. In cultivars exhibiting the shortest average incubation periods (12-13 days), only a few isolates caused more than 20% infection, except cultivar BAT 332 (8 isolates). It would appear that these cultivars were resistant to most isolates tested and very susceptible to just a few.

Table 12. Disease incubation period in 21 cultivars inoculated with 17 isolates of *Phaeoisariopsis griseola*[a].

Cultivar	Incubation period (days) Average[b]	range[c]	No. of isolates inducing 20% or more disease severity
G 5686	19	14-23	7
Amendoin	18	14-20	6
A 235	17	12-22	2
BAT 1647	17	10-23	3
Caraota 260	17	13-20	2
Calima	17	11-21	13
G 2858	17	13-22	6
Cornell 49242	17	14-19	4
G 1805	16	12-22	6
A 62	16	11-21	9
Seafarer	16	12-20	10
Alabama No.1	16	11-20	8
Montcalm	15	12-19	14
Pompadour Checa	15	11-17	5
Jalo EEP 558	14	10-20	13
A 301	14	10-19	9
A 339	13	13-13	1
BAT 332	13	10-18	8
A 212	12	12-12	1
BAT 76	12	10-13	2
A 21	12	11-13	3

[a]Incubation period was expressed as number of days after inoculation to develop 20% disease severity.

[b]Average number of days calculated over the number of isolates inducing 20% or more disease.Six plants per cultivar were evaluated.

[c]Minimum and maximum incubation period exhibited by each cultivar to develop 20% disease severity.

Pathogenic variation in 17 P. griseola isolates is summarized in Table 13. The data show that Montcalm was the most susceptible cultivar while cultivar A 339 exhibited the widest spectrum of resistance. No cultivar was immune to all isolates of the pathogen.

The twenty-one bean cultivars differed in the number of isolates to which they were susceptible. The arbitrary classification shown (Table 13) shows that some cultivars had similar patterns of reaction to the 17 isolates used. It also shows that isolates could be grouped in different pathogenicity groups according to the cultivars infected, ie. Colombia 1 and Guatemala 1.

Eight of the 21 cultivars were selected for use in an attempt to develop a uniform set of differential cultivars. Cultivars selected were those exhibiting uniform and consistent disease reactions, differential reactions to the several isolates used in the present studies, differences in seed size and color, and no more than one intermediate reaction to an isolate.

The eight differential cultivars selected were Montcalm, Seafarer, G 5686, BAT 332, Pompadour Checa, Cornell 49242, BAT 1647, and A 339. Other characteristics were considered in selecting this set of cultivars. For example, cultivar Montcalm was universally susceptible to all pathogen isolates while cultivar Seafarer was able to differentiate isolates of low pathogenicity (infecting only 4 or 5 cultivars) from isolates of high pathogenicity (infecting 11 or more cultivars). Cultivar G

Table 13. Disease reactions of 21 bean cultivars inoculated with 17 Latin American isolates of Phaeoisariopsis griseola[a].

Cultivars	Isolates of P. griseola[b]																
	Co 1	Gu 1	Me 1	Gu 2	Ar 1	Br 1	Br 3	CR 1	Co 2	Br 2	Br 4	Co 3	Co 4	Ni 1	Ar 2	Ar 3	Ar 4
Montcalm	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Jalo EEP 558	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S
Calima	S	S	S	S	S	S	S	S	I	I	S	S	S	S	S	S	S
Amendoin	S	S	S	S	S	S	I	S	S	S	I	S	S	S	I	S	S
G 2858	S	S	S	S	S	S	S	S	S	S	S						
Seafarer	S	S	S	S	S	S	S	S	S	S	S						
Alabama No 1	S	S	S	S	S	S	S	S	S	S	I						
A 62	S	S	S	S	S	S	S	S	I	S	S						
G 5686	S	S	S	S	S	S	S	S				S	S				
G 1805	S	S	S	S	S	S	S	I		I	S						
A 21	S	S	S	S	S	S	I	I	I	I							
BAT 332	S	S	S	S	S	S	S			S	S						
A 301	S	S	S	S	S	S	S			S	S						
A 212	S	S	I	S	I	S	I	S	I								
A 235	S	S	I	S	S	S	I		I								
BAT 76	S	S	I	I	S	S	I										
Caraota 260	S	S	I	S				S	I	I							
Pompadour Checa	S	S	S	S				S	S								
Cornell 49242	S	S	S	S					S								
BAT 1647	S	S			S	I	S										
A 339	S	S															
No cultivars infected	21	21	19	19	17	17	17	14	14	13	11	5	5	4	4	4	4

[a] S: susceptible, > 4% leaf area covered by ALS lesions
I: intermediate, 1-4% leaf area covered by lesions
blank: resistant, < 1% leaf area covered by lesions

[b] Country and identification number; Colombia (Co), Guatemala (Gu), Mexico (Me), Argentina (Ar), Brazil (Br), Costa Rica (CR), and Nicaragua (Ni).

5686 differentiated isolates of low pathogenicity belonging to two different groups; cultivar BAT 332 was always severely infected by Brazilian isolates while cultivars Pompadour Checa and Cornell 49242 were always resistant to the Brazilian isolates. Cultivars BAT 1647 and A 339 were highly resistant to most isolates, but very susceptible to isolate Colombia 1.

Pathogenicity Groups in 42 P. griseola Isolates from Latin America and Africa Using a Differential Set of Eight Bean Cultivars.

Forty-two (42) isolates of P. griseola were divided into fourteen pathogenicity groups on the basis of their reactions on the eight differential bean cultivars (Table 14). Most of the pathogenicity groups contained several different isolates much as conventional races. No cultivar was immune to all P. griseola isolates. However, cultivar BAT 1647 was resistant to isolates in 11 of the 14 pathogenicity groups.

Four of the pathogenicity groups contained isolates from both Latin America and African countries, while the remaining 10 groups contained isolates from only one or the other continent. The most as well as the least pathogenic groups included isolates from Latin America and Africa. Pathogenicity groups 7 and 14 included the largest number of isolates from different countries (Table 14).

Pathogenic variation was noted for isolates both within and between different African countries. For example, the four

Table 14. Pathogenicity groups in Phaeoisariopsis griseola.

Pathogenicity group and isolate			Disease reaction on host cultivar*							
			Montcalm	Seafarer	BAT 332	Pompadour Checa	G 5686	Cornell 49242	A 339	BAT 1647
1	A	Colombia 1	S	S	S	S	S	S	S	S
	B	Guatemala 1	S	S	S	S	S	S	S	S
	C	Tanzania 2	S	S	S	S	S	S	S	S
2	A	Uganda 1	S	S	I	S	S	S	R	I
	B	Uganda 2	S	S	I	S	S	S	R	S
	C	Zaire 2	S	S	I	S	S	S	R	S
3	A	Mexico 1	S	S	S	S	S	S	R	R
	B	Guatemala 2	S	S	S	S	S	S	R	R
4	A	Kenya 2	S	S	S	S	R	S	S	R
5	A	Brazil 1	S	S	S	R	S	R	R	I
	B	Brazil 3	S	S	S	R	S	R	R	S
	C	Argentina 1	S	S	S	R	S	R	R	S
6	A	Rwanda 3	S	S	R	S	S	S	R	R
	B	Burundi 3	S	S	R	S	S	S	R	R
7	A	Tanzania 1	S	I	S	S	R	R	S	R
	B	Kenya 1	S	I	I	S	R	R	S	R
	C	Uganda 3	S	S	I	S	R	R	I	R
	D	Rwanda 1	S	S	S	S	R	R	S	R
	E	Burundi 1	S	S	S	S	R	R	S	R
	F	Burundi 2	S	I	S	S	R	R	S	R
	G	Zaire 1	S	S	S	S	R	R	S	R
8	A	Colombia 2	S	S	R	S	R	S	R	R
9	A	Costa Rica 1	S	S	R	S	S	R	R	R
	B	Rwanda 2	S	S	R	S	S	R	R	R
10	A	Brazil 2	S	S	S	R	R	R	R	R
	B	Brazil 4	S	S	S	R	R	R	R	R
11	A	Tanzania 3	S	S	R	R	R	R	S	R
	B	Malawi 2	S	S	R	R	R	R	S	R
12	A	Malawi 5	S	R	S	R	R	R	S	R
	B	Malawi 9	S	R	S	R	R	R	S	R
13	A	Colombia 3	S	R	R	R	S	R	R	R
	B	Colombia 4	S	R	R	R	S	R	R	R
	C	Malawi 3	S	R	R	R	I	R	R	R
14	A	Malawi 4	S	R	R	R	R	R	R	R
	B	Malawi 7	S	R	R	R	R	R	R	R
	C	Malawi 10	S	R	R	R	R	R	R	R
	D	Tanzania 4	S	R	R	R	R	R	R	R
	E	Kenya 2	S	R	R	R	R	R	R	R
	F	Nicaragua 1	S	R	R	R	R	R	R	R
	G	Argentina 2	S	R	R	R	R	R	R	R
	H	Argentina 3	S	R	R	R	R	R	R	R
	I	Argentina 4	S	R	R	R	R	R	R	R

*S= Susceptible. > 4% leaf area covered by P. griseola lesions.
I= Intermediate, 1-4% leaf area covered by lesions.
R= Resistant, < 1% leaf area covered by lesions.

isolates from Tanzania were classified into four separate pathogenicity groups, including the most and least pathogenic. Isolates from Malawi were generally among the least pathogenic African isolates.

Cultivar BAT 1647 was highly resistant to isolates from both Latin America and Africa and was infected only by isolates in highly pathogenic groups 1,2, and 5. On the other hand, cultivar A 339 was highly resistant to most Latin American isolates but was susceptible to several African isolates, including isolates in the slightly pathogenic groups (Table 14). Cultivar Montcalm was susceptible to all isolates from both continents, indicating that this cultivar could be considered universally susceptible.

The only pathogenicity groups containing a single isolate were groups four (Kenya 3) and eight (Colombia 2). Five groups contained only African isolates while three groups contained only Latin American isolates.

Proposed pathogenicity groups for Phaeoisariopsis griseola are presented in Table 15. Isolates that induced susceptible or intermediate disease reactions were both classified as susceptible (S) to facilitate interpretation of the results.

Differences in virulence in terms of disease severity and aggressiveness were observed among isolates of all pathogenicity groups. Isolates caused the same amount of disease severity on one cultivar, yet differed in length of incubation period (Tables 16,17). For example, isolate Tanzania 2 required 8 days to produce 20% disease severity on cultivar G 5686 while isolate

Table 15. Proposed pathogenicity groups in Phaeoisariopsis griseola*.

Host cultivar	Pathogenicity group													
	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	R	R	S	R	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 49242	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

*S= Susceptible, 1% or more leaf area covered by P. griseola lesions
R= Resistant, < 1% leaf area covered by lesions

Colombia 1 required 21 days (Table 17).

Virulence of an isolate was again independent of pathogenicity. Isolates from group 14 were as virulent as isolates from group 1 in terms of disease severity and aggressiveness on the same cultivar.

Aggressiveness of isolates within a particular pathogenicity group was cultivar dependent. For example, isolates in pathogenicity group 5 were very aggressive on cultivar BAT 332, however the same isolates needed an additional 11 days to produce 20% disease severity on cultivar G 5686. Isolates from pathogenicity groups six and eight, however, were of very low aggressiveness on all eight bean differentials (Table 17).

Cultivars BAT 332 and A 339 were generally associated with decreased aggressiveness of the pathogen. These two cultivars exhibited low levels of disease when inoculated with isolates of pathogenicity groups two, seven, and 12, which contain only isolates from Africa.

In general, isolates that produced 20% of disease in less than 13 days after inoculation, were highly virulent in terms of final disease severity on the same cultivars.

Table 16. Disease severity in 8 bean cultivars inoculated with 42 isolates of *Phaeoisariopsis griseola*[a].

Pathogenicity group and isolate			Montcalm	Seafarer	BAT 332	Pompadour Checa	G 5686	Cornell 49242	A 339	BAT 1647
1	A	Colombia 1	48	33	50	50	23	50	43	50
	B	Guatemala 1	40	22	40	35	40	40	14	18
	C	Tanzania 2	50	50	38	50	50	50	16	40
2	A	Uganda 1	50	13	5	50	33	50		5
	B	Uganda 2	50	12	3	50	50	43		14
	C	Zaire 2	50	50	4	50	50	50		15
3	A	Mexico 1	40	50	50	50	25	5		
	B	Guatemala 2	6	20	40	25	16	33		
4	A	Kenya 2	50	11	15	50		17	36	
5	A	Brazil 1	40	40	43		18			3
	B	Brazil 3	40	50	43		20			25
	C	Argentina 1	45	10	42		30			25
6	A	Rwanda 3	40	40		34	30	45		
	B	Burundi 3	42	33		27	39	50		
7	A	Tanzania 1	50	4	8	50			15	
	B	Kenya 1	50	5	1	13			10	
	C	Uganda 3	50	16	4	50			6	
	D	Rwanda 1	50	50	15	50			16	
	E	Burundi 1	50	30	16	8			7	
	F	Burundi 2	50	4	9	7			11	
	G	Zaire 1	50	10	16	50			9	
8	A	Colombia 2	25	40		27		30		
9	A	Costa Rica 1	40	25		40	8			
	B	Rwanda 2	50	50		50	50			
10	A	Brazil 2	6	29	19					
	B	Brazil 4	18	25	39					
11	A	Tanzania 3	50	25					50	
	B	Malawi 2	50	38					19	
12	A	Malawi 5	50		6				8	
	B	Malawi 9	50		9				11	
13	A	Colombia 3	50				43			
	B	Colombia 4	50				47			
	C	Malawi 3	50				5			
14	A	Malawi 4	39							
	B	Malawi 7	50							
	C	Malawi 10	31							
	D	Tanzania 4	43							
	E	Kenya 2	30							
	F	Nicaragua 1	32							
	G	Argentina 2	40							
	H	Argentina 3	40							
	I	Argentina 4	50							

[a]Disease severity expressed as percentage of leaf area covered by ALS lesions. Maximum disease severity = 50%, minimum disease severity (blank) = 0%.

Table 17. Number of days after inoculation to produce 20% of disease severity in 8 bean cultivars inoculated with 42 isolates of Phaeoisariopsis griseola*.

Pathogenicity group and isolate			Days Required to Develop 20% Disease Severity							
			Montcalm	Seafarer	BAT 332	Pompadour Checa	G 5686	Cornell 49242	A 339	BAT 1647
1	A	Colombia 1	11	15	11	11	21	18	13	11
	B	Guatemala 1	18	15	15	15	19	16		21
	C	Tanzania 2	8	11	14	8	9	8		16
2	A	Uganda 1	13			14	18	17		
	B	Uganda 2	11			11	13	16		
	C	Zaire 2	13	14		12	14	18		
3	A	Mexico 1	14	11	12	11	15			
	B	Guatemala 2		16	16	13		19		
4	A	Kenya 2	9			9			12	
5	A	Brazil 1	13	12	11		22			
	B	Brazil 3	16	16	13		21			25
	C	Argentina 1	16		11		20			20
6	A	Rwanda 3	17	18		19	20	18		
	B	Burundi 3	18	19		20	18	18		
7	A	Tanzania 1	11			18				
	B	Kenya 1	12							
	C	Uganda 3	11			12				
	D	Rwanda 1	9	15		12				
	E	Burundi 1	11	22						
	F	Burundi 2	10							
	G	Zaire 1	11			14				
8	A	Colombia 2	19	16		19		19		
9	A	Costa Rica 1	19	18		17				
	B	Rwanda 2	14	26		14	18			
10	A	Brazil 2		20						
	B	Brazil 4		15	18					
11	A	Tanzania 3	10	16					17	
	B	Malawi 2	8	14						
12	A	Malawi 5	11							
	B	Malawi 9	13							
13	A	Colombia 3	20				14			
	B	Colombia 4	20				16			
	C	Malawi 3	11							
14	A	Malawi 4	17							
	B	Malawi 7	10							
	C	Malawi 10	16							
	D	Tanzania 4	16							
	E	Kenya 2	19							
	F	Nicaragua 1	13							
	G	Argentina 2	16							
	H	Argentina 3	15							
	I	Argentina 4	14							

*blanks: disease severity of 20% was not reached.

DISCUSSION

The literature on bean angular leaf spot disease is somewhat sparse compared to other major bean diseases. However, interest in ALS has increased during the last few years due to its increasing importance in Latin American and African bean production areas where many of the local commercial varieties have been severely damaged. Continued attempts to develop resistant cultivars require studies of pathogen variability and host resistance.

The present studies were focused on variability of the pathogen as it relates to identification and development of resistant host germplasm. Numerous studies provide evidence that plant pathogens are extremely variable in pathogenicity and virulence. Most of these studies consider pathogenicity a qualitative trait, where differences are based on ability to infect or not infect a common host. On the other hand, virulence is considered a quantitative trait whereby differences are mainly based on severity of disease produced on the same host.

The results presented here provide evidence that P. griseola varies considerably both in pathogenicity and virulence. Isolates interacted differentially with host varieties. Some of the isolates induced very susceptible reactions on some bean cultivars, whereas, on other cultivars the same isolates were non-pathogenic.

Variation in pathogenicity was evident among isolates from different countries as well as isolates within a country. No cultivar was immune to all isolates tested. These differences among isolates explain earlier observations in several CIAT annual reports that varietal reactions varied from country to country as well as from season to season in the same locality.

A high degree of host-pathogen specificity between bean cultivars and isolates of P. griseola was evident in these studies. The effects of resistant genes present in the cultivars used were dependent on the pathogen isolate. This phenomenom is usually observed when major genes condition resistance which is termed race specific or vertical resistance (18,21,42).

The most pathogenic isolates were from Jutiapa (Guatemala), and Santander de Quilichao (Colombia). The latter site is where CIAT's Bean Pathology program evaluates the Bean Angular Leaf Spot International Nursery. Such an arrangement suggests that resistant cultivars selected at this site will probably be resistant in other Latin American countries.

The 17 Latin American isolates could be separated into two major groups. Group one contained isolates of high pathogenicity and group two contained isolates of low pathogenicity. The fact that the first group contained isolates from six different countries suggest that good sources of resistance might be selected within each country, provided the field evaluations are conducted at locations where highly pathogenic isolates exist.

Few studies have been carried out on the variation among

isolates of plant pathogens as related to virulence characteristics or parasitic fitness attributes. Most of the studies are also based on just a few or one cultivar. Results of the present studies indicate great differences in parasitic fitness among isolates. Quantitative differences observed were supported by the degrees of disease severity induced by each isolate. High disease severity was observed for isolates of very low pathogenicity, which allows further classification of isolates within pathotypes or races. Table 4 indicates that these less pathogenic isolates caused no infection on most cultivars, however, they were able to induce at least 40% disease severity on at least one bean cultivar. This was confirmed by the lack of correlation between the pathogenic ability of the isolates and the disease severity induced by them.

The results clearly indicate differences in virulence in terms of disease severity among isolates. The range of infection on the 21 bean cultivars inoculated with each Latin American isolate of the pathogen also indicates that amount of disease produced by each isolate was cultivar dependent.

Disease severity was measured as percentage of leaf area covered by lesions. This area will depend either on the number of lesions or the size of the lesions or both. These results indicated a significant positive correlation (r: 0.79) between average number of lesions and disease severity. However, the number of lesions produced by a particular isolate is also dependent on the cultivar used. According to Table 5, the range

of number of lesions produced by each isolate on the infected cultivars is highly variable.

Clear differences in average lesion size induced by P. griseola were observed. However, this factor was not correlated with disease severity (r: -0.25), and a low negative correlation (r: -0.49) was observed between lesion size and number. For most isolates (Table 6), lesion size appeared cultivar dependent.Small lesions were induced only by isolates Colombia 4, Nicaragua 1, Argentina 3, and Argentina.

A pathogen isolate would be considered highly virulent if a large number of reproductive spore cycles develop during the life cycle of the host. This attribute could be considered a measure of the pathogen's aggressiveness. The fewer the number of days needed by a pathogen to produce a given level of disease, the more reproductive cycles the pathogen may have.

Results indicate that isolates varied in aggressiveness. Very important is the fact that disease severity and aggressiveness (as measured by number of days required to produce 20% disease), were significantly negatively correlated (r: -0.71). This indicates that isolates producing a 20% amount of disease within the shortest time also produced a larger amount of disease. However, the range of aggressiveness indicated in Table 7 shows that interactions exist between isolates and cultivars. For example, isolate Colombia 1 which was very aggresive, needed 22 days after infection to produce 20% of disease severity on some bean cultivars.

The last component of virulence studied was spore production per unit area of lesion. The results indicated differences among isolates in their ability to produce spores. Interactions between isolates and cultivars were noted.

In addition, there was no correlation between spore production and other components of virulence such as disease severity, number of lesions, lesion size, and incubation period.

This lack of correlation leads to several conclusions. First, the characterization of a highly virulent isolate under greenhouse conditions does not necessarily mean that it would induce epidemic levels of disease in the field, particularly if secondary inoculum production is low. Second, the characterization of an isolate with high index of sporulation per unit area does not indicate a potential for epidemic disease development since the pathogen isolate might possess a low infection frequency. In other words, only a small proportion of spores landing on susceptible tissue would result in lesions. Third, differences among isolates in sporulation could have been due to artifacts of the experimental methods. Fourth, the optimum time when spores are formed within a lesion is not known. Perhaps studies of spore production should be based on cumulative spore production over a period of time.

Further detailed studies on sporulation in P. griseola are warranted, including improved methods for spore collection. Such studies should also define optimum conditions of humidity and temperature for spore production , since these factors are

important to the formation of fungal reproductive structures.

Nelson (29) indicates that resistance in plants can be classified as of two types. The first type is one in which the infection process is restricted, thereby impeding succesful establishment of a parasitic relationship. This resistance is also referred as hypersensitivity, specific resistance, vertical resistance, or major gene resistance. In the second type of resistance, the host restricts colonization and growth of the pathogen following infection. Such resistance is also termed nonspecific_resistance, horizontal resistance, field resistance, and minor gene resistance.

Cultivars are considered to possess genes for specific resistance if those genes confer resistance to some races of the pathogen but not to others (29). On the other hand, Vander Plank (43) stated that plants having non-specific resistance show low levels of disease severity and are thought to have some degree of effectiveness against all races of the pathogen. According to Nelson (29) this kind of resistance functions by reducing the rate of disease increase which can result from host mechanisms that retard penetration, increase latent period, restrict amount of tissue colonized, and reduce the amount and duration of sporulation.

The results obtained in these studies have confirmed previous reports (12,13) on the broad resistance of cultivars A 235, BAT 76, A 339, and BAT 1647 to ALS in Latin America. The present study also revealed that these four cultivars exhibited

susceptible reactions to particular pathogen isolates obtained in the same country. For example, the four cultivars reacted differentially to the four Colombian isolates; cultivars A 339 and BAT 1647 were resistant to all isolates from Brazil, but cultivars BAT 76 and A 235 reacted differentially to the same isolates.

The susceptible reaction of cultivar BAT 332 reported consistently from Brazil and resistant reaction for the same cultivar in Popayan, Colombia (12,13), was confirmed in these studies. The cultivar was susceptible to isolates from three separate locations in Brazil and resistant to isolates obtained from Popayan, Colombia. However, BAT 332 was susceptible to isolates from the Santander de Quilichao location in Colombia, which has not been reported.

All but one of the reported resistant reactions for the cultivars used in these studies were confirmed in artificial greenhouse inoculations. The only exception was cultivar Jalo EEP 558, reported as highly resistant at different locations in Brazil and Colombia (13). This cultivar proved susceptible to all 17 isolates used for the greenhouse studies. In all cases, seed of the differential cultivars used to characterize pathogenicity groups was derived from single plant selections. Thus, the possibility was eliminated that seed mixtures were responsible for the contrasting greenhouse results. It is known that virulence of pathogens and plant growth and development are greatly influenced by environment. Thus, it is important to

carefully control inoculum concentration, stage of host development, and other environmental variables.

The results of this study and previous reports clearly suggest that the highly resistant cultivars possess specific resistance. However, the presence of specific resistance does not preclude the concomitant presence of non-specific resistance in the same cultivar. Van der Plank, in fact suggests that race-non-specific resistance is probably always present along with race-specific resistance (43).

Non-specific resistance effective against many races of a pathogen is difficult to detect. Little is known as to how to select most efficiently for non-specific resistance. Van der Plank (43) indicates that lesion size, number of lesions per plant, lesion color, latent period, and number of conidia per lesion are factors that could be used to identify non-specific resistance.

The present studies examined components of non-specific resistance such as disease severity, lesion number, lesion size, incubation period, and sporulation capacity. Considerable variation among cultivars for each one of these components was found. The high resistance to ALS in some of the cultivars reported in the literature could be due to the operation of one or more of these components.

Several researchers have used the mean value of disease reactions, slopes of the regression lines and deviations from regression to clarify cultivar-pathogen relationships (10).

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Cultivars exhibit greater resistance to all isolates of a pathogen if means are low, regression coefficients are zero, and the deviations from regression are minimal. The authors feel that these analyses provide a better understanding of host-pathogen associations.

The regression analyses on disease severity for the 21 bean cultivars on the 17 P. griseola isolates indicated that none of the cultivars was resistant to all isolates of the pathogen. The slope of the regression line was significantly different from zero for all cultivars containing a low mean of disease severity, and deviations from regression were minimal. This indicates the presence of the differential interaction (specific resistance) of these cultivars with some of the isolates. However, it is possible that these cultivars may have broadly based resistance to most isolates while being susceptible to a few.

Cultivars A 339, A 212, BAT 76, BAT 1647, A 235, and Caraota 260 showed very low disease severities, which indicate that these cultivars may reduce the infection frequency (relation between number of spores landing on the plant surface and number of lesions developed). This phenomenon is a component of resistance normally associated with horizontal resistance (32), in which a higher concentration of spores are required for infection of resistant cultivars as compared to susceptible.

Cultivars A 339, and Cornell 49242 showed high degrees of resistance in terms of low lesion numbers. The regression lines

[redacted]

showed a slope not significantly different from zero, small deviations from regression, and low mean values for lesion numbers, indicating non-specific resistance for this particular component.

A significant positive correlation between disease severity and lesion numbers indicates that resistant cultivars could be selected by counting numbers of lesions. However, the fact that there was no correlation between lesion number and lesion size indicates selection based on lesion numbers is not always effective. For example, a cultivar may develop low numbers of lesions and yet the lesions are large leading to the assignment of a susceptible reaction. Such was the case for cultivar G 2858 which had low numbers of lesions but lesion size was as large as those on the most susceptible cultivars. On the other hand, a cultivar such as Amendoin may have small lesion size but large number of lesions, also resulting in a susceptible reaction.

Among all 21 cultivars, A 339 was the only one to exhibit low disease severity, low number of lesions, and small lesion size. This cultivar, together with BAT 1647, had low mean values for lesion size, slopes did not differ significantly from zero, and deviations from regression were minimal. Therefore, These two cultivars show presence of several of the components of non-specific resistance (Tables 9,10,11).

Several studies have reported differences in non-specific resistance components among cultivars differing in their degree of resistance. While the components may be independent, it would

be possible to recombine them to increase the level of resistance of a particular cultivar (32).

Host resistance involving long latent or incubation periods is one of the most effective strategies to reduce rate of disease development. Studies have shown a direct relationship between host genotype resistance and latent period. As a result of the extended latent period, a pathogen will complete fewer reproduction cycles during the growing season, resulting in less disease severity (32).

Incubation period was arbitrarily defined as number of days after inoculation until 20% disease severity was evident. One disadvantage of this definition is that it does not permit one to make correlations between incubation period and other components of non-specific resistance; this is so because not all isolates of the fungus produce 20% disease severity on all cultivars. Consequently, it might have been more useful to determine the number of days after inoculation until 50% of lesions contained spores. Nevertheless, the results indicated that some cultivars such as G 5686 exhibit large amounts of disease but only after long incubation periods. Such a phenomenon would lead to slower disease development.

Analyses of multiple regression equations, which predict the operation of horizontal resistance from both the latent period and the infection frequency, suggest that differences in non-specific resistance in the field are largely explained by differences in latent period, rather than differences in

infection frequency (32). Thus, latent period may be a good parameter for predicting horizontal resistance. An example is G 5686 in which long incubation periods were observed.

Cultivar A 339 which exhibited a high degree of resistance in terms of lesion size and lesion number, developed more than 20% disease with only one pathogen isolate. Unfortunately, the incubation period was 13 days indicating a high degree of susceptibility to that particular pathogen isolate. On the other hand, none of the other isolates of the fungus caused more than 20% disease severity which indicates that A 339 possesses a broad and high level of resistance.

Resistance in several cultivars was expressed on the basis of incubation period (Table 12). This resistant component could then be recombined with other components to increase the overall level of resistance.

Latent period is a critical component determining the apparent infection rate when a large number of reproductive cycles of the pathogen are required to develop a disease epidemic. The fewer the number of reproductive cycles, the more important the effect of the other components of resistance will become. The disease cycle for the angular leaf spot disease is between 9 to 12 days under favorable conditions. The life cycle of a bean plant can last 90 to 120 days depending on the growth habit of the cultivar and environmental conditions. This means that P. griseola could complete up to 10 conidial generations on a susceptible cultivar in just one growing season.

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Therefore, incubation period should be an important component to be considered in selecting for resistance to angular leaf spot. Detection of cultivars exhibiting long incubation periods under field conditions should be performed during the early stages of infection since alloinfection or infection arising from spores produced by neighboring susceptible plants would interfere with efficient selection of resistant plants.

Disease progression is directly proportional to inoculum availability; inoculum is, of course, dependent on sporulation capacity. Reduction of sporulation capacity is another important component of horizontal resistance, however, the measurement of spore production is difficult from a technical standpoint. Nevertheless, differences in spore production among isolates were observed in the present study. Comparisons among cultivars were not possible due to the rapid defoliation in several susceptible bean cultivars.

Specific studies on the ability of bean cultivars to affect pathogen sporulation should be examined under more controlled conditions. Sporulation is highly dependent on humidity and temperature. Generally, lesion size and sporulation capacity are positively correlated. Smaller lesions produce fewer spores, which reduces the rate of pathogen spread (42). If such a correlation is found between lesion size and sporulation capacity, the difficulties of estimating sporulation capacity could be overcome by measuring lesion size (10,32).

In many cases however, lesion size may not be correlated with

spore production. Techniques for measuring spore production could lead to interfering with the cumulative sporulating process and result in erroneous interpretations. In addition, Parlevliet (32) indicates that at high pustule density, carbohydrate production by the leaf is reduced. Under such conditions the pustules compete with the leaf for scarce nutrients which results in a negative correlation between spores per pustule and pustule size, or pustule density. Such negative interferences operate not only at high, but also at moderate pustule densities. He recommends that comparison of spore production in various genotypes should be done at similar infection frequencies, which is diffficult to achieve experimentally.

Parlevliet (32) further suggested that, in host-pathogen systems where the resistance and the degree of pathogenicity vary quantitatively, the host resistance and level of pathogenicity of the pathogen are important. In such cases, the dependency and hence interaction between resistance and level of pathogenicity are to be expected. He points out that the interaction of components of non-specific resistance and parasitic fitness indicates that non-specific resistance could erode to some extent over time.

A search for bean genotypes which reduce the apparent infection rate of ALS should receive greater attention, although the stability of this rate-reducing capacity remains to be determined. The implications of the interaction found between components of non-specific resistance and parasitic fitness would

mean that the widespread use of cultivars with rate-reducing capacity could lead to natural selection in the pathogen population for increased fitness, thus limiting the stability of the host resistance.

Van der Plank (43) indicates that specific resistance is more likely to be successful when, for ecological reasons, direct adaptation of the pathogen to the host is restricted, as it is with stem rust of wheat in North America. Specific resistance is almost certain to be temporary when direct adaptation is favored, as occurs with most diseases in the tropics and subtropics where host plants are found through the entire year.

The 42 P. griseola isolates from Latin American and African countries were divided into fourteen pathogenicity groups on the basis of their reactions on a set of eight bean cultivars. Both the most as well as the least pathogenic groups included isolates from both continents, while intermediate groups included isolates either from one or both continents.

From a breeding point of view, it is advantageous that the most pathogenic groups contained isolates from several different countries from the two continents. However, isolates belonging to the same pathotype or race when tested on a few differentials may be separated when more differentials are used. Williams and Owen (44) arranged 10 isolates of Rhynchosporium secalis into 8 pathogenicity groups under fluctuating temperature conditions. However, under controlled conditions, only two races were differentiated among 122 isolates. Only further studies will

determine whether this could also be the case for several of the pathogenicity groups established for P. griseola.

The results of these studies show that great pathogenic variation also exists in isolates of P. griseola from Africa. Those cultivars exhibiting more resistance to the Latin American isolates were also more resistant to the African isolates and those more susceptible to the first group of isolates were also more susceptible to the second.

The eight bean cultivars selected as differentials were very useful in separating P. griseola isolates into different pathogenicity groups. These differentials are available at the CIAT's Bean Pathology Program.I suggest that these cultivars be used by CIAT as well as other different bean researchers working with the ALS disease. CIAT could be responsible for increase and distribution of the bean differentials. The differentials were originally selected as pure lines and would make comparisons meaningful among pathotypes identified and reported from different localities in the world.

The use of differential varieties with known genes for resistance to P. griseola is essential. It is recommended that specific studies be conducted with the bean differentials used in these studies to determine the number and type of resistance genes involved.

It appears that resistance in cultivars A 339 and BAT 1647 is non-specific in nature and under polygenic control. However, Nelson (30) concluded that the control of horizontal resistance

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ranges from a single gene to many genes. In some cases resistance is thought to be polygenic until tests using more controlled conditions to reduce the nonheritable variance revealed that resistance is monogenic. If the nonheritable variance exceeds heritable variance, the former could mask the latter resulting in a continuous distribution of phenotypes even if the genetic variability for resistance conditions discontinuity.

Of primary importance is whether the use of one isolate of the pathogen is sufficient to identify resistance to the spectrum of variability in the pathogen population. It is known that if a pathogen exhibits wide pathogenic variation the effectiveness of single-isolate screening is reduced.

These studies indicate that Santander de Quilichao in Colombia is a good site to screen for ALS resistance under field conditions. Highly pathogenic isolates have been obtained at this site and it is likely that a resistant cultivar selected here would be resistant at many other localities. Fortunately, CIAT is screening germplasm at this locality, and the presence of the BALSIT in common trials with the germplasm screened allows one to detect highly pathogenic isolates present during all growing seasons. On the other hand, CIAT formerly screened for ALS resistance at Popayan, Colombia. These results revealed that pathogen isolates from Popayan were less pathogenic than isolates from Santander.

Likewise, the localities of Milungui in Tanzania and Jutiapa in Guatemala should be useful sites to select bean cultivars with

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broadly based resistance to ALS. The common use of the eight bean differentials recommended here should allow the identification of the best localities in each country to conduct tests for ALS resistance.

It is not possible to discuss the relative parasitic fitness of a pathogen genotype without referring to the host genotype used to make the test. Likewise, reference to the relative resistance of a host genotype should be made only with regard to the particular pathogen genotype on which the comparison was made.

Data on the interaction between specific host genotypes and specific pathogen isolates is necessary to ensure selection of the most appropiate type of resistance for a particular situation.

ALS resistance can obviously be most efficiently studied by international cooperative tests, in which cultivars with a broad spectrum of resistance are identified and utilized.

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CHAPTER II

ISOLATION AND PARTIAL CHARACTERIZATION OF TOXIC METABOLITES PRODUCED IN CULTURE BY *Phaeoisariopsis griseola*

INTRODUCTION AND LITERATURE REVIEW

Plant pathogens have long been known to produce toxic substances involved in pathogenesis. Many toxic compounds are produced by microorganisms in culture, however only a few are recognized as determinants of disease (16). Toxins are classified as host-specific (12,18) and non-host-specific (9,18). Host-specific toxins affect only plants infected by the pathogen, while non-host-specific toxins affect both host plants and non-host plants of the producing organism (7).

Yoder (23) considers host-specific toxins as pathogenicity factors and non-host-specific toxins as virulence factors. The term pathogenicity factor is a qualitative term and means a pathogen-produced molecule that is required for disease initiation and tissue colonization. The term virulence factor refers to molecules that are not necessary for disease initiation but contribute to virulence or are required for certain symptom development. Virulence relates to amount of disease, and is quantitative in nature.

Toxin-producing pathogens have played an important role in

agriculture by virtue of their destructive effects and their contribution to epidemic development in a number of crops. Prime examples are the southern leaf blight of corn during 1970 and 1971 and the victoria blight disease of oats, which caused vast losses in North America during 1946-1948. Both diseases are caused by fungi in the genus Helminthosporium and both pathogens produce host-specific toxins (16,18). Non-specific toxins have also been important factors in disease epidemics, of which the best example is bean halo blight caused by Pseudomonas syringae pv.phaseolicola (7,16). Chlorotic symptoms associated with infection are apparently the result of toxin action.

At least 15 host-specific toxins are now recognized in six fungal genera; non-specific toxins known to be involved in disease are also few in number (16). The most common genera producing host-specific toxins are Helminthosporium and Alternaria while Pseudomonas is the most common bacterial genus producing non-host selective toxins.

According to Scheffer (16), toxins are products of microbial pathogens, cause obvious damage to plant tissue, and are known with confidence to be involved in disease development. In order to understand the molecular basis of certain diseases and disease resistance, it is important to identify, isolate, characterize chemically, and to determine the mode of action of toxins.

A number of guidelines have been proposed to ascertain the significance of toxins in disease (23). Among these are:

isolation of toxin from diseased plants, reproduction of typical disease symptoms when toxin is applied to healthy plants, correlation of the host-specificity of the pathogen and toxin production, and correlation of virulence with ability to produce toxin. However, proof of a role for toxins in disease can be difficult (17). The most powerful tools for evaluation of the role of a toxin in plant diseases are those involving mutant organisms, biochemical techniques, and genetic analyses of the pathogen, host or both (16,17,23).

Host-selective toxins induce all of the visible and physiological effects that are induced in plants by the infecting fungi (15). On the other hand, non-host-specific toxins are known to cause only a portion of disease symptoms (7). Some of the most common symptoms caused by toxins are: increases in respiration and ion leakage, decreases or increases in protein synthesis, uptake of several solutes by the host cell, and dark fixation of CO_2 (16).

None of the diseases involving host-specific toxins have been shown to fit the complex gene for gene pattern as described by Ellingboe (4,16,18). Instead, data supports the hypothesis that these toxins have receptor or sensitive sites in the susceptible cell, and that such sites are lacking or have less affinity for toxin in the resistant cell (16,18). The plasma membrane is most often suggested as the receptor site, however, conclusive evidence is lacking (16,23).

Mode of action of non-host-specific toxins is better

understood than the mode for host-specific toxins. In most cases these toxins are potent enzyme inhibitors (7). Pseudomonas syringae pv. tabaci produces tabtoxin, a toxin that inhibits glutamate synthetase; P. s. pv. phaseolicola produces phaseolotoxin, which inhibits the the enzyme ornithine carbamoyltransferase; Rhizobium japonicum produces rhizobitoxine which inhibits B-cystathionase; and Alternaria tenuis produces tentoxin which inhibits coupling factor 1 in chloroplasts. All of these toxins are associated with chlorosis development in leaves of certain plants. However, the biological significance of rhizobitoxine is not known. It is also not clear if there are other sites of action for toxins in vivo, as well as how the biochemical lesions caused by these toxins lead to chlorosis in infected leaves.

Once the role of a toxin is suspected in a plant disease, further research requires dependable methods for the production and isolation of the toxic compound. Because toxins are products of microbial metabolism, detecting techniques used to monitor their production and isolation are similar to those used for other microbial products such as antibiotics, vitamins, and enzymes. Common methods of separation include chromatography of various types (TLC, HPLC, GC, gel filtration, and ion exchange).

Two requirements for critical studies of natural products are availability of a reliable assay, and demonstration of purity (20). Lack of sensitive and reliable assays for toxins have on many occasions made the difference between success and failure

(16).

Ideally, highly purified toxin should be used for critical studies and activity expressed on a dry weight basis; however, this is difficult to achieve with toxins that are labile during purification procedures (16). Toxins may be very active yet labile, may be bound and inactivated in plant tissues, or toxin effects may be masked by other metabolic products of the pathogen (15,16).

In addition, the purification process should be carried out until homogeneous toxin is obtained; results based on research with impure compounds have led to erroneous interpretations (20). Trace amounts of a very active material might accidentally be copurified with a major component that has different or no biological activity. Partially purified materials however, are adequate for preliminary work (20).

Angular leaf spot lesions caused by P. griseola are necrotic areas surrounded by chlorotic halos. Complete yellowing of infected leaves followed by premature defoliation normally occurs. Since chlorosis formation and necrotic lesion formation have been associated with several toxin producing organisms, the present study was conducted to elucidate the possible involvement of a toxin in the development of ALS. Preliminary experiments showed that liquid culture filtrates of the fungus contained toxic metabolites which induce necrotic and chlorotic spots when bioassayed on an ALS susceptible bean cultivar. Several chromatographic techniques were used in attempts to isolate and

purify a toxin from culture filtrates of several P. griseola isolates.

MATERIALS AND METHODS

Fungal Isolates and Plant Material

Cultures of P. griseola used for toxin production included the single spore isolates of cultures Michigan 5, Kenya 2, Malawi 7, and Colombia 1 as described previously. Inocula for use in toxin studies were obtained from cultures mantained on V-8 juice agar for 10-12 days at 24 C.

Bioassays were conducted on the youngest trifoliolate leaves of plants 17-22 days old. Plants were grown in the greenhouse or growth chamber at 24 to 30 C in flats containing either vermiculite or a 2:1 mixture of soil and vermiculite. Plants were fertilized weekly with 20-20-20 soluble fertilizer.

Toxin Production

In preliminary experiments, flasks (125 ml) containing 25 ml of a modified Fries medium containing yeast extract (11), or liquid V-8 juice (200 ml V-8 juice, 3g $CaCO_3$, 800 ml distilled water) were inoculated with a 5x5 mm^2 plug of each pathogen isolate grown on V-8 juice agar. Three flasks each for isolates Michigan 5, Kenya 2, and Malawi 7 were inoculated; flasks were incubated at room temperature for 12, 16, 20 or 24 days.

For producing larger amounts of toxin, one liter Roux bottles containing 200 ml of modified Fries medium were inoculated and incubated at room temperature for 20 to 24 days.

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Culture fluids were harvested by filtration through several layers of cheesecloth, followed by filtration through Whatman No. 1 filter paper. Five to ten liters of crude culture filtrates were concentrated to approximately 10% of original volume under reduced pressure at 35 C. An equal volume of methanol was added to deproteinize the suspension which was then stored overnight at 4 C. Precipitates were removed by filtration, and the methanol evaporated from the solution under reduced pressure to a final volume of approximately 2.5% of original. The concentrated aqueous solution obtained to this point was used directly for gel filtration or for extraction with different solvents prior to further attempts at purification as indicated in the results.

Toxin Bioassays

The most common method used for bioassay of solutions was the leaf puncture method similar to that used by Stermer et al (22). Excised trifoliolate leaves were punctured with a group of eight entomological needles wrapped together by a parafilm band. A 10 ul drop of test solution was immediately placed on the puncture sites. One to three different tests were performed on each side of the midrib depending on the experiment. Treated leaves were placed on top of glass beads contained in 9 cm petri dishes; 10 ml water was placed in the botton of the dishes. Petri plates were then covered with a black cloth, which was removed 48 hours later. Lesions developing at the puncture sites were measured at 24 or 48 hr after treatment.

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Electrolyte leakage was determined using 2 cm diameter leaf discs. Two-tenths gm fresh weight of leaf discs selected at random from different leaves were placed in cheesecloth bags and submerged in 10 ml of test solutions or water contained in scintillation vials. The samples were vacuum infiltrated for 20 min, and then allowed to incubate one additional hour in the test solutions.

Vials were then emptied and the leaves carefully rinsed in distilled water. An additional 10 ml distilled water was added to each vial and samples were allowed to incubate at room temperature for 30 min. Conductivity measurements were made hourly with a Markson conductivity meter containing a pipet-type electrode (K=1). Two replications were used for each treatment and both the susceptible cultivar Montcalm and the resistant cultivar BAT 1647 were used.

The root growth inhibition bioassay was performed by placing 10 ml of test solutions or water into 9 cm petri dishes. Five seeds of each cultivar per replication were placed into the test solutions for three to four days at which time root lengths were recorded. Two replications were used per treatment.

Gel Filtration and Solvent Extractions

Molecular exclusion chromatography or gel filtration was conducted with Sephadex G-25 (1.4x70 cm) or Sephadex G-15 (1.4x90 cm) columns. Concentrated toxin solutions were loaded onto the columns in 1 ml amounts and eluted with distilled water at a flow

rate of 0.1 ml per minute. Eighty (80) fractions in amounts of 2-5 ml were collected from the columns and assayed directly for toxicity as described previously. Fractions showing toxicity were combined, concentrated, and used in other purification steps; dry weights and dilution end points were determined on these pooled samples.

The concentrated, deproteinized, aqueous toxin preparations were partitioned three times with each of several solvents of increasing polarity such as hexane, chloroform, methylene dichloride, ethyl acetate, and water saturated n-butanol. The solvent extracts were combined, reduced to near dryness, and resuspended in several mililiters of distilled water. Toxicity was determined for each solvent extraction by a leaf bioassay. Toxin-containing fractions were used for further purification and determination of properties such as lability, dry weight, and dilution end point.

Adsorption Chromatography

Adsorption chromatography on straight-phase C_{18} (40 um, J.T. Baker Chemical Co.) column (2.2x15 cm) was performed for all toxin samples prior to HPLC. Loaded samples were simultaneously eluted with 100 ml each of : H_2O, 10%, 20%, 30%, 50%, and 100% ethanol. Collected fractions were reduced to near dryness under reduced pressure and dissolved in 1 ml of distilled water. Presence of toxic compounds in the fractions was determined with the leaf bioassay technique. Toxic fractions were then used for

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Adsorption chromatography was also performed on a straight-phase silica (230-400 mesh, 40-63 um) flash column (1.0x15 cm). Toxin samples were loaded and eluted with acetone at a flow rate of 5 cm/min. Five ml fractions were collected, reduced to near dryness and dissolved in 500 ul of water. Activity of the fractions was then determined by leaf bioassay.

High Pressure Liquid Chromatography (HPLC)

Toxic fractions obtained from the C_{18} column were chromatographed on a Waters uBondapac C_{18} HPLC column (.78x30 cm) using a Varian 5000 machine. A 100 ul sample of aqueous toxin was injected into the port. Water was used as solvent A and ethanol as solvent B. Samples were eluted with a gradient of ethanol linearly changed from 0% to 100% in a 60 min period plus a constant 10 min period with 100% ethanol. The flow rate was constant at 1.0 ml/min and a UV detector monitored absorption at 220 nm at the outlet of the column. Fractions were collected, the ethanol was removed under reduced pressure at 35 C, and samples bioassayed for the presence of toxins.

Thin Layer Chromatography (TLC)

TLC was performed on 0.25 Merck Silica Gel 60 plates. Samples loaded on the plates were developed with single or mixtures of solvents of different polarity. Substances on the plates were assayed either for toxin or exposed to long and short

waves of UV light for detection of fluorescent materials. For detection of toxic activities, plates were cut in 0.5 cm bands and separately scraped into test tubes. The silica powder was extracted with acetone or distilled water, the extracts reduced to dryness, suspended in 0.5 ml distilled water and bioassayed.

Several visualizing reagents were tested as reported by others (2,14). Such reagents included iodine (alkaloids), ninhydrin (aminoacids), anisaldehyde (carbohydrates), bromcresol green (carboxilic acids), potassium permanganate (diterpenoids), vanillin (terpenes), and the epoxide indicator 4-(p-nitrobenzyl)-pyridine (epoxide groups). Both long (366 nm) and short (254 nm) wavelengths of ultraviolet (UV) light were also used to visualize compounds.

RESULTS

TOXIC METABOLITES PRODUCED BY P. griseola IN CULTURE.

Toxin Bioassys

The leaf-puncture bioassay was used throughout these studies. Though considered somewhat variable, the method was very practical for bioassaying large numbers of individual fractions collected through different chromatographic techniques. A major problem with this method involved reproducibility of the lesion diameters. However, the problem was minimized by the use of at least four replications per treatment. Specificity was determined in several cases by the diameter of the lesions, since dilution end point assays revealed just 10-fold differences in sensitivity to toxin between cultivar Montcalm and BAT 1647.

Better symptoms, including more severe chlorosis, were obtained when leaves used in bioassays were from young plants (17-22 days old) as compared to those from older plants.

Data from electrolyte leakage tests and root growth inhibition bioassays were inconclusive, therefore the data are not presented. The major problem with electrolyte leakage tests was that the controls with water showed early and high loss of electrolytes for both susceptible and resistant cultivars. Another problem with this method was the large amount of toxin needed for the bioassay.

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The major problem with the root growth inhibition bioassay was uneven seed germination of the test cultivars. Differences in root growth between a large seed type (Montcalm) and a small seed type (BAT 1647) made it difficult to detect differences in growth due to toxin activity. Different pigments released by the seeds into the suspension medium during bioassays (red from Montcalm and black from BAT 1647) might have influenced the results; the pigments might have been toxic themselves, or might have interfered with the toxicity of filtrates.

Preliminary Experiments

The results in Table 18 indicate that P. griseola produced compounds in vitro that were toxic to P. vulgaris leaves. The fungal metabolites were more toxic in culture filtrates after 20 to 24 days of growth than after 12 to 16 days of growth. Toxic compounds were detected in the modified Fries medium but not in the V-8 liquid medium (Table 18) when cultures were grown for 12, 16, and 20 days. Mild symptoms were induced from culture filtrates after 24 days growth on V-8 liquid medium.

Lesions were produced on leaves of both susceptible and resistant cultivars when culture filtrates 16 or 20 days old were assayed. However, filtrates from 12 and 24 days old growth induced lesions only in the susceptible cultivar. Lesions were always larger in the susceptible cultivar than in the resistant (Table 18). From this point on culture filtrates were obtained from cultures grown on modified Fries medium for 20 to 24 days.

Table 18. Lesions induced on leaves of Montcalm and BAT 1647 bean cultivars by toxic metabolites present in culture filtrates of three Phaeoisariopsis griseola isolates[a]

Isolate	Cultivar and Reaction[b]	Media[c]							
		Fries medium				V-8 medium			
		Culture Age (days)				Culture Age (days)[d]			
		12	16	20	24	12	16	20	24
Kenya 2	BAT 1647 (R)	-	4	5	-	-	-	-	4
	Montcalm (S)	11	6	10	14	-	-	-	4
Malawi 7	BAT 1647 (R)	-	4	4	-	-	-	-	4
	Montcalm (S)	6	6	13	15	-	-	-	4
Michigan 5	BAT 1647 (R)	-	4	5	-	-	-	-	4
	Montcalm (S)	6	6	17	15	-	-	-	4

[a]Lesion diameter in millimeters. -= no lesion.
[b]Bean cultivar and ALS reaction; R= resistant, S= susceptible.
[c]125 ml flasks containing 25 ml of Fries medium or V-8 medium were inoculated with a small plug of agar containing mycelium and spores of each isolate of the pathogen.
[d]Inoculated flasks were incubated in stationary culture for 12,16, 20, and 24 days. Presence of toxic metabolites in culture filtrates were determined by a leaf-puncture bioassay.

Symptoms induced by the fungal metabolites included necrotic spots surrounded by a chlorotic halo at the bioassay sites. These symptoms were larger, yet very similar to those associated with P. griseola infections. Lesions produced by the toxic metabolites generally were seen four days after treatments while the fungus requires at least 10 days to produce the lesions in vivo.

Controls in which sterile non-inoculated media were assayed produced no symptoms. Contamination and/or saprophytic growth were frequently observed in leaves tested with filtrates. Lesions formed at these contaminated sites were watersoaked rather than necrotic with halos, and were seen only after four days.

The toxic metabolites detected in culture filtrates of P. griseola were relatively unstable. Activity was lost by autoclaving for twenty minutes or after three days exposure at room temperature. Toxicity was also lost when original culture filtrates were diluted greater than 1:10. Toxicity was not reduced by storage of crude culture filtrates for three months at 4 C or at -20 C.

Toxicity was specific, but not in all cases, for the susceptible cultivar Montcalm (Table 18). In other cases, lesions were exhibited by both susceptible and the resistant cultivars; however, lesions were consistently larger for susceptible Montcalm than for resistant BAT 1647 cultivar.

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Gel Filtration and Solvent Extractions

Toxic metabolites produced by two isolate of P. griseola in culture were purified by using column chromatography (gel filtration) with both Sephadex G-25 (exclusion limit for molecular weight of 5,000) and Sephadex G-15 (exclusion limit for molecular weight of 1,500). Isolates Michigan 5 (pathogenic on cultivar Montcalm) and Colombia 1 (pathogenic on both Montcalm and BAT 1647) were used.

Michigan 5 culture filtrate was concentrated to 0.1 of original volume, deproteinized with an equal volume of methanol, then concentrated about ten more times. One ml of the final sample was passed through a Sephadex G-25 column from which twenty fractions of five ml each were eluted with water and tested for activity using the leaf bioassy. Lesions similar to those produced by the angular leaf spot (ALS) pathogen were observed in the susceptible cultivar Montcalm four days after bioassay. Fractions showing activity were Nos. 16 to 18 (80 to 90 ml eluted volume). None of the other collected fractions were toxic.

Two ml each from fractions 13 to 20 were individually concentrated to 200 ul and tested for activity. Symptoms were first observed 48 hours after treatment with fractions 16 to 18 (Figure 3) at most biossay sites in the susceptible cultivar. After four days leaves treated with fractions 14 and 15 exhibited mild chlorosis (3-5 mm in diameter), but these lesions dit not enlarge thereafter. Lesions produced by fractions 16,17, and 18

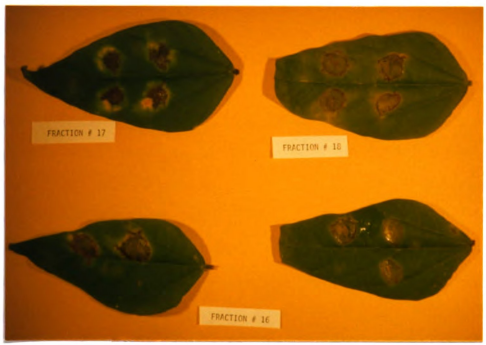

Figure 3. ALS susceptible bean cultivar Montcalm exhibiting necrotic and chlorotic symptoms induced by toxic fractions Nos. 16 to 18 (80 to 90 ml eluted volume). Concentrated toxic solutions (1 ml) of isolate Michigan 5 were loaded onto a Sephadex G-25 column (1.4x70 cm) and eluted with water at a flow rate of 0.1 ml/min. Eighty fractions (5 ml each) were collected and bioassayed by the leaf-puncture method. Each of the toxic fractions 16 to 18 were concentrated further 10 fold and bioassayed again by placing a 10 ul drop at four different sites on bean leaves.

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averaged 12, 8, and 11 mm diameter, respectively. The calculated dry weights of these three fractions were: 16, 4mg/ml; 17, 1.8 mg/ml; 18, 0.5 mg/ml.

Chromatography of toxic samples with a Sephadex G-15 column did not increase activity on a weight basis in the eluted fractions (Table 19). Each fraction collected from the column was more concentrated on a dry weight basis than fractions collected from a Sephadex G-25 column; however the lowest activity had a dilution end point in the range of 3 to 9 mg/ml for the susceptible cultivar in four different experiments. Activity was increased, however, in terms of time required for expression of symptoms. Lesions appeared 24-48 hours after bioassay on the susceptible cultivar and at least 12 hours later on the resistant cultivar. Toxic fractions were never collected near the void volume of the Sephadex G-15 column, suggesting that the toxic substances are of low molecular weight (< 1,500).

Culture filtrates from isolate Colombia 1 were concentrated and extracted with methanol as described above before being passed through a Sephadex G-15 column. Toxic substances were detected by leaf bioassays in a large number of fractions collected. Symptoms developed on both Montcalm and BAT 1647 cultivars when the toxic fractions were bioassayed. Necrotic spots and chlorotic halos developing at the bioassay sites were similar and somewhat larger than those typically seen in ALS-infected leaves. This suggests that the toxic metabolites produced by isolate Colombia 1 are of a different nature than

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Table 19. Active fractions (AF), dry weight (DW), and dilution end point (DEP) of Michigan 5 toxic metabolites collected after passage through a Sephadex G-15 column[a].

	AF[b]	DW of pooled fractions (mg/ml)[c]	DEP[d]	
			S	R
Experiment 1	42-43	60	1/10	1/1
Experiment 2	46-49	83	1/10	1/1
Experiment 3	44-65	555	1/100	1/10
Experiment 4	41-55	357	1/100	1/10

[a]1 ml of a Michigan 5 culture filtrate previously concentrated 10 times, extracted with an equal volume of methanol, and concentrated 10 times was passed through a Sephadex G-15 column and eluted with water. 2 ml fractions were collected in each of four separate experiments.

[b]Active fractions induced necrosis and chlorosis in bioassays on susceptible cultivar Montcalm, similar to symptoms of the ALS disease.

[c]Active fractions were pooled and concentrated to about 10 ml.

[d]Dilution end points of the pooled fractions exhibiting symptoms on the susceptible (S) cultivar Montcalm and on the resistant (R) cultivar BAT 1647.

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those produced by isolate Michigan 5 which showed some degree of specificity for the susceptible cultivar Montcalm.

Crude culture filtrates of the same isolate Colombia 1 also showed activity on both cultivars Montcalm and BAT 1647; both cultivars are also susceptible to this particular isolate.

Repeated partitioning of aqueous toxin solutions with hexane, chloroform, methylene dichloride, ethyl acetate, or n-butanol extracted varying amounts of toxic compounds, depending upon the isolate.

Isolates of P. griseola pathogenic only on cultivar Montcalm yielded most of their toxic metabolites with selectivity towards the same cultivar in the chloroform or methylene dichloride extractions. The most diluted sample showed activity at a concentration of 0.1 to 1 mg/ml, which indicates a small increase in purity of the preparation via solvent extraction. The remaining fractions extracted with more polar solvents were active on both cultivars Montcalm and BAT 1647 and gave dilution end points in the range of 10 to 100 mg/ml. Gel filtration of chloroform or methylene dichloride extracts gave similar elution patterns as those obtained initially for aqueous solutions of the toxin.

In contrast to the Michigan 5 isolate, toxic activity in culture filtrates of isolate Colombia 1 was found in most of the solvent extractions used above. Compounds extracted with chloroform or methylene dichloride were active mainly on cultivar Montcalm while those extracted with ethyl acetate or n-butanol

were active on both cultivars. In all cases, lesions were similar to those caused by P. griseola. This was not the case for the ethyl acetate and n-butanol fractions obtained from isolates pathogenic only on Montcalm, which induced mainly watersoaked lesions.

These results suggest that isolates such as Michigan 5 produced one kind of toxic compound with selectivity towards cultivar Montcalm. On the other hand, isolate Colombia 1 produced several toxic compounds, some of which are specific for Montcalm, and some which are active on both Montcalm and BAT 1647 cultivars.

Adsorption Chromatography on C_{18} and Flash Silica Gel Columns

Adsorption of low polarity compounds in the active fractions obtained from Sephadex G-15 or solvent extractions was carried out on a 2.2x15 cm column of C_{18}. Loaded samples were simultaneously eluted with 100 ml each of: H_2O, 10%, 20%, 30%, 50%, and 100% ethanol. Collected fractions were concentrated at 35 C to approximately 1 ml under reduced pressure.

Toxic compounds were detected in the 50% ethanol extracts in all cases. Activity in the toxic fractions was increased ten fold by the use of the C_{18} column (Table 20); toxicity was detected in the range of 10 to 60 ug/ml.

The toxic fractions were again selective for the Montcalm cultivar with isolate Michigan 5 or with isolate Colombia 1 extracted with methylene dichloride. Non selective toxicity was

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Table 20. Dry weights and dilution end points (DEP) for toxic fractions of two P. griseola isolates extracted with 50% ETOH from a C_{18} column.[a]

Isolate[b]	dry weight mg/ml	DEP of activity on cultivar[c] Montcalm	BAT 1647
Michigan 5[d]			
exp 1	6.0	1/100	1/10
exp 2	2.0	1/100	1/10
exp 3	4.0	1/100	1/10
Colombia 1[e]			
MC 1	5.6	1/100	1/10
MC 2	1.2	1/100	1/10
nB 1	5.0	1/100	1/100
nB 2	1.5	1/100	1/100

[a] C_{18} columns were loaded and eluted with equal volumes of H_2O, and 10%, 20%, 30%, 50%, and 100% ETOH simultaneously. Toxic compounds were present in the 50% ETOH extracts.

[b] Michigan 5 is pathogenic on Montcalm; Colombia 1 is pathogenic on Montcalm and BAT 1647.

[c] Highest dilution of active fractions still inducing necrosis and chlorosis in leaf bioassays.

[d] Experiment 1 was a fraction extracted with methylene dichloride, experiments 2 and 3 were pooled active fractions from a Sephadex G-15 column.

[e] MC 1 and MC 2 are two separate fractions extracted with methylene dichloride from culture filtrates of isolate Colombia 1. nB 1 ans nB 2 are two separate fractions extracted with n-butanol from culture filtrates of isolate Colombia 1.

found for the n-butanol extracts of isolate Colombia 1 that were passed through the C_{18} column and eluted with 50% ethanol.

Nonselective toxicity was always observed for the water extracts. However, the symptoms developed at the bioassay sites were different from those induced by the 50% ethanol extracts, and not similar to ALS. Moreover, activity was lost at 1/10 dilutions and dry weights of these fractions were in the range of 300 to 900 mg/ml. This indicates that probably most of the compounds present in the water fractions are contaminants.

All toxic fractions obtained from gel filtration, solvent extractions, or chromatography on C_{18} lost activity within a few days when aqueous solutions were stored either at room temperature, 4 C or -20 C. This suggests that the toxic compouns produced by P. griseola are very labile during purification procedures.

Collected fractions from flash chromatography on silica were assayed for toxic activity; in addition, fractions were processed by thin layer chromatography (TLC) using UV light as indicator for the presence of fluorescent spots. Chromatography of a toxin sample obtained from isolate Colombia 1 yielded several fractions with differing specificities for cultivars Montcalm and BAT 1647.

The first fractions with toxic activity eluted from the silica column exhibited specificity for cultivar Montcalm; the fractions chromatographed at rf: 0.87 and 0.75 when TLC plates of silica gel were developed with acetone and exposed to UV light.

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The next group of eluted fractions showed selectivity for cultivar BAT 1647 and chromatographed at rf: 0.69. The last group of fractions were active on both Montcalm and BAT 1647 and chromatographed between rf: 0.57 and 0.45.Active fractions were found in the first 180 ml eluted from the same flash silica column in subsequent tests. Concentration studies indicated that the toxic compounds were at concentrations less than 100 ug/ml.

Fluorescent areas on TLC plates never resulted in toxicity when the areas were removed, extracted with acetone or water, and bioassayed on leaves. This may have been due to low amounts of toxic compounds recovered from the TLC plates or to loss of activity during the extraction process.

High Pressure Liquid Chromatography (HPLC)

Toxic compounds in the 50% ethanol extracts from a C_{18} column were further purified by HPLC. Numerous compounds were detected in the column eluates as indicated by UV absorption patterns at 220 nm; the compounds were detected in eluates of both Michigan 5 and Colombia 1 isolates.

Several active peaks which caused lesions on cultivar Montcalm were detected in fractions collected from isolate Michigan 5. Active peaks (peaks 1,2,3, Figure 4) were small peaks compared to other larger peaks (peaks A,B,C, Figure 4) which showed no activity. The minor peaks eluted from the column when the concentration of solvent B (ETOH) was running at a concentration of 20 to 55% and had retention times of 12, 19, and

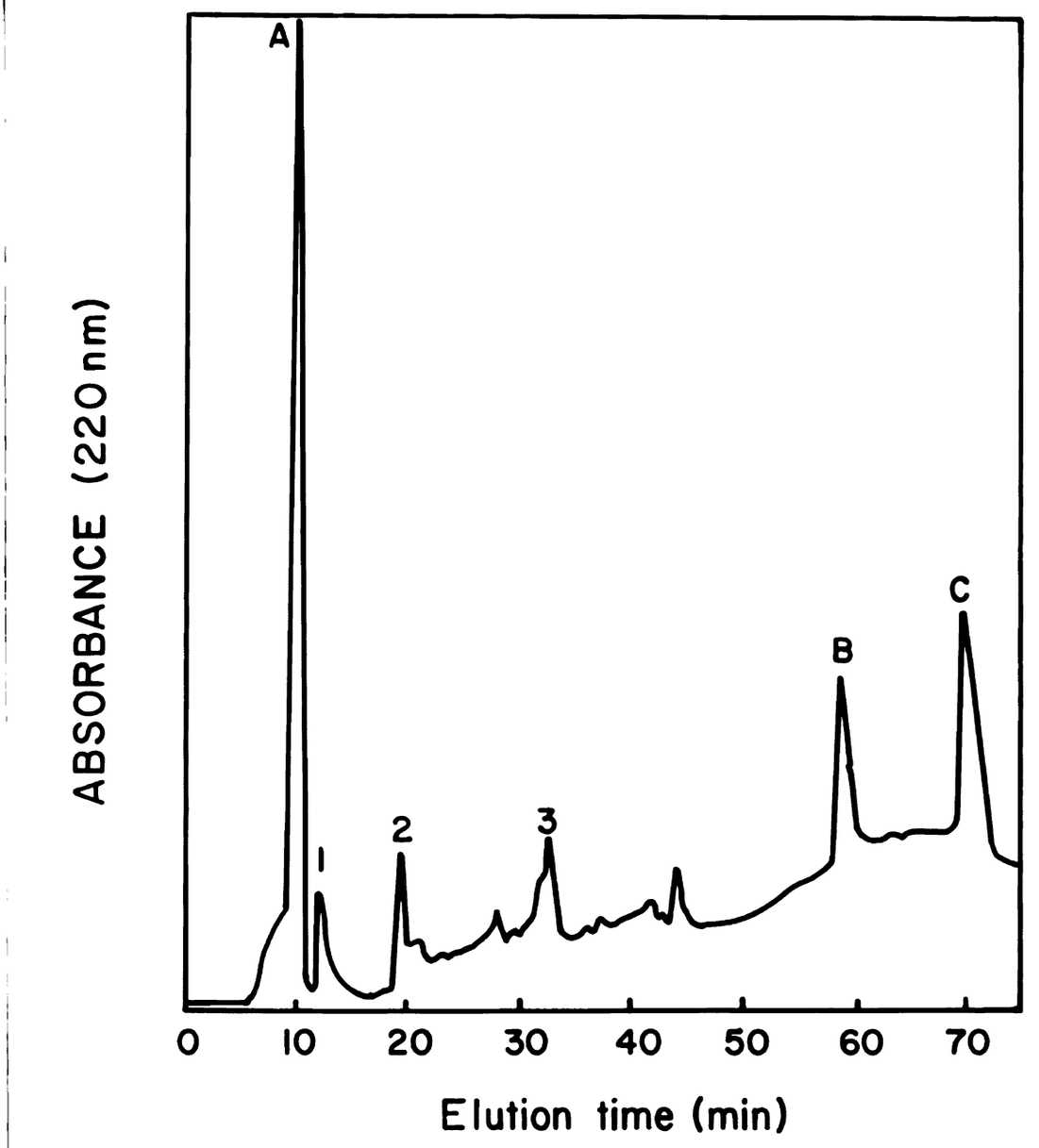

Figure 4. HPLC elution profile of toxic (1,2,3) and non-toxic (A,B,C) peaks eluted from reverse phase C_{18} column. The solvent system used consisted of water and ETOH. A linear gradient of 0 to 100% ETOH was obtained in 60 minutes at 1 ml/min. Elution continued at 100% ETOH for 10 more minutes. A 100 ul sample of Michigan 5 toxic fraction eluted from a C_{18} column with 50% ETOH was injected into the HPLC.

32 minutes respectively. Active peaks 1,2, and 3 yielded other minor peaks when rerun individually through the HPLC.

The large non toxic peaks A,B, and C which contained almost 100% of the total amount of sample (2 mg) injected into the HPLC probably represent major contaminants present in the injected sample.

Thus the active toxic fractions collected were probably at concentrations on the order of micrograms or even nanograms. Further concentration of the active fractions was not attempted.

Toxicity in the active fractions was lost when they were stored in aqueous solutions at 4 C or at -20 C for one week, indicating that the toxic compounds become increasingly labile with increased sample purity.

Isolate Colombia 1 yielded several peaks when the 50% ethanol extracts were run on HPLC. All active peaks were eluted from the column when the concentration of solvent B (ETOH) was running at a concentration of 30 to 60% (Figure 5).

Of the fractions collected, peaks 3 and 4 (retention times 20 and 21 minutes respectively) showed selectivity for cultivar BAT 1647; peak 10 (retention time 33 minutes) showed selectivity for cultivar Montcalm, and peaks 7 and 8 (retention time 28 and 29 minutes, respectively) showed toxicity on both cultivars. The remaining peaks (Figure 5) were not toxic.

Active peaks 3,4,7,8, and 10 yielded other minor peaks when samples were rerun individually through HPLC. These minor peaks were not assayed for toxic activity.

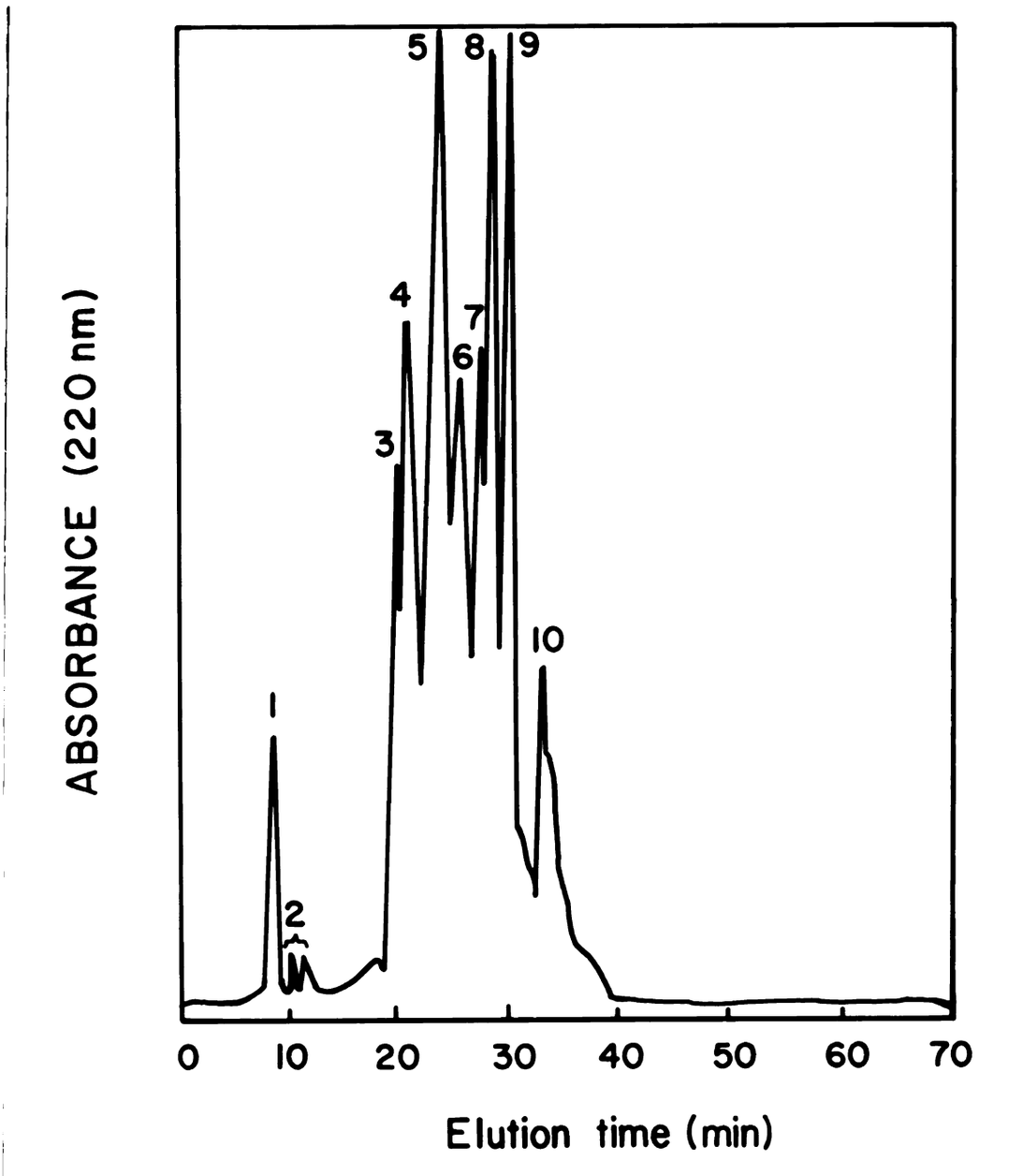

Figure 5. HPLC elution profile of toxic (3,4,7,8,10) and non-toxic (1,2,5,6,9) peaks eluted from reverse phase C_{18} column. The solvent system used consisted of water and ETOH. A linear gradient of 0 to 100% ETOH was obtained in 60 minutes at a flow rate of 1 ml/min. Elution continued at 100% ETOH for 10 more minutes. A 100 ul sample of Colombia 1 toxic fraction eluted from a C_{18} column with 50% ETOH was injected into the HPLC.

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As with isolate Michigan 5, toxic fractions from isolate Colombia 1 collected from HPLC lost activity when aqueous solutions were stored for one week at 4 C or -20 C.

Thin Layer Chromatography (TLC)

Further purification of the toxic metabolites produced by P. griseola was not possible by TLC. It was not possible to correlate spots detected on TLC plates with toxic activity. This was because 10 ul of the toxin solution, which is easily detected in a leaf bioassay , overloaded the plate and produced poor resolution and streaking when sprayed with different reagents or when exposed to UV light. Large amounts of impurities found with the toxin in the HPLC purification step probably explains the poor resolution.

Efforts to recover toxic material was also not succesful when a TLC plate was divided in several bands of 0.5 cm, the silica gel removed, extracted with acetone or water, dried under reduced vacuum, and dissolved in water.

The best resolution with TLC plates plates was obtained for fractions collected from a flash chromatography column with silica. However, toxicity could not be associated with detected spots since recovery of toxin from TLC plates was not possible.

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DISCUSSION

Many different fungi and bacteria produce substances toxic to plants, and at least some of these substances are disease determinants (17). Most of the toxins known today that are involved in the disease process have initially been detected on the basis of toxicity to certain host plants of cell free culture filtrates. However, culture filtrates of almost every microorganism are frequently toxic to plants (17) and a careful evaluation of their role in disease is necessary.

The present experiments indicate that P. griseola produces toxic compounds under certain cultural conditions of growth. Cell free culture filtrates obtained from three different pathogen isolates grown on a modified Fries medium exhibited similar toxicity when tested on the same host cultivar. Toxic metabolites were not produced in a V-8 liquid medium. The Fries medium has been used in numerous other studies relating to toxin production by fungal plant pathogens.

Several observations suggest possible involvement of these pathogen produced toxic compounds in the ALS disease. First, culture filtrates of the three different isolates of P. griseola were selectively toxic towards the cultivar Montcalm which is highly susceptible to all three isolates. Much less toxicity was observed on the resistant cultivar BAT 1647 depending on age of the culture; however, toxicity in terms of lesion diameter was

always less in the resistant than in the susceptible cultivar. Selective toxicity of culture filtrates towards the same genotypes that are infected by the producing organisms was involved in the discovery of several well known host-selective toxins.

Secondly, induction of typical disease symptoms by small amounts of crude culture filtrates have led to the recognition that toxic compounds may play a role in disease. However, "typical" visible symptoms may also be induced by factors not involved in disease (23). Toxins also can cause either typical or atypical symptoms, depending on factors such as concentration and assay procedures. Thus symptoms may not be a reliable criteria for evaluating the possible role for a toxin in disease, according to Yoder (23).

Nevertheless, induction of symptoms similar to ALS was used in the present study as a measure of toxicity in preparations. Necrotic areas surrounded by chlorotic halos were induced by crude culture filtrates of three different isolates on the susceptible cultivar Montcalm; these symptoms resemble those associated with the ALS disease. In contrast, atypical lesions were induced by water extracts during several stages of the purification process, which consisted of large watersoaked areas with irregular borders and without any necrosis and chlorosis.

Another good indication that a toxic compound detected in crude culture filtrates is involved in pathogenesis relates to the high biological activity of such compounds. Culture filtrates

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from Helminthosporium victoriae still caused root growth inhibition in susceptible oats even at a 1:10^{6} dilution, while a 1:10 dilution did not affect roots of the resistant variety (6). Such high biological activity plus the selectivity implies a role for this toxin in the host-pathogen interaction.

Such a high degree of specificity is however not typical for most of the known host-specific toxins. Crude culture filtrates of P. griseola which induced large lesions on the susceptible cultivar lost most of this biological activity at 1:10 dilutions in distilled water. The loss of activity is difficult to explain, although lability is a common problem for many biological compounds. Some host-specific toxins are notably unstable, and living plant tissue may completely inactivate a toxin (19).

It is interesting that culture filtrate of isolate Colombia 1 which is pathogenic to both Montcalm and BAT 1647 cultivars, was also toxic to both cultivars. This suggests that the toxic metabolites produced by isolate Colombia 1 are different from those produced by isolates Michigan 5, Malawi 7, and Kenya 2.

Conventional column chromatography was used to initially purify toxic compounds in culture filtrates. Gel filtration studies with a Sephadex G-15 column indicated that the toxic compounds are of molecular weight lower than 1,500, which is very similar to the size of most toxins involved in plant pathogenesis. Activity of the toxic fractions eluted from the column was not enhanced on a dry weight basis. Activity was found in the range of 3 to 9 mg/ml which is still considered very

high for a possible toxin involved in disease.

The concentration needed to induce symptoms in the resistant cultivar was just 10 times greater than that required to produce symptoms on the susceptible cultivar. The low biological activity found in these toxic fractions could be explained in part by the poor resolving power of gel filtration for compounds with similar molecular weights. Moreover, toxic fractions may have contained different non toxic contaminants which are responsible for the high dry weight data. It is also possible that the toxic compounds are highly labile and activity was lost during dilution.

Biological activity on a weight basis increased approximately 10 fold when crude culture filtrates were concentrated by extraction with different organic solvents. Interestingly, toxic compounds with some selectivity towards cultivar Montcalm were extracted with the less polar solvent methylene dichloride from culture filtrates of the two different pathogenic isolates Michigan 5 and Colombia 1. On the other hand, more polar solvents such as n-butanol extracted toxic compounds from Colombia 1 but not from Michigan 5 culture filtrates. These n-butanol extracted compounds induced typical ALS symptoms on both cultivars Montcalm and BAT 1647. These results suggest that isolate Colombia 1 probably produces two separate groups of toxic compounds. One group may be similar to the toxic compounds produced by isolate Michigan 5 and are more soluble in less polar solvents and show selectivity towards the susceptible cultivar

Montcalm. The other group is soluble in more polar solvents, but not present in culture filtrates of isolate Michigan 5; these toxic compounds are active on both cultivars Montcalm and BAT 1647.

The simultaneous presence of both host-selective and non-host selective toxic products in culture filtrates of fungi is relatively common. The host-specfic toxins of Helminthosporium carbonum, a pathogen of corn, and the toxins of of H. victoriae, a pathogen of oats, are accompained by less toxic non-specific metabolites, which damage both susceptible and resistant plants (10,13). The host-specific toxin of H. carbonum caused inhibition of root growth of the susceptible cultivar at 0.5 ug/ml but required 50 ug/ml for root growth inhibition of the resistant cultivar. This selective toxin was isolated from culture filtrates by extractions into chloroform. The non-specific toxin, called carbtoxinine, was equally toxic to root tissues of both the susceptible and the resistant cultivars, causing growth inhibition at 25 ug/ml. This non-selective toxin was extracted into butanol.

The non-specific toxin produced by H. carbonum has a different host range than the pathogen , and is not detectable in crude culture filtrates. The toxic compounds extracted with n-butanol from culture filtrates of isolate Colombia 1 have the same host range as the producing isolate. Unfortunately, there is no bean cultivar resistant to isolate Colombia 1 which would allow us to further test the specificity of these compounds.

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same host range as the producing isolate. Unfortunately, there is
no bean cultivar resistant to isolate Colombia 1 which would
allow us to further test the specificity of these compounds.

The observations indicate that P. griseola produces some toxic compounds which may be important for pathogenicity in some cultivars and other toxic compounds important for pathogenicity to other cultivars. This interpretation is supported by data obtained from chromatography through a C_{18} column of the solvent extractions of isolates Michigan 5 and Colombia 1 culture filtrates; active concentration was between 10 and 60 ug/ml.

Toxic fractions in methylene dichloride extracts of Michigan 5 or Colombia 1 culture filtrates required a concentration 10 times greater to produce the same lesions in the resistant cultivar BAT 1647 than the concentration required to produce lesions in the susceptible cultivar Montcalm based on DEP studies. On the other hand, the same minimun concentration was equally toxic on both cultivars when the toxic fractions were obtained from the n-butanol extractions of Colombia 1 culture filtrates. This same effect on both cultivars indicates no selectivity and possibly involvement of these toxic compounds in the pathogenicity of Colombia 1 on both cultivars.

It is common during purification procedures to detect more than one component having biological activity. Generally, it is not known whether these compounds represent analogs, intermediates in the synthesis and/or degradation of a single toxin, or whether they are unrelated compounds (3).

Although analogs of the H. carbonum toxin are found in culture filtrates, they are less active than the major toxin (14). The toxin of H. victoriae losses activity when treated with

$NaHCO_3$, and two different components appear in paper chromatographs (5); one of these components (victoxinine) is toxic but much less so than the original toxin preparations and exhibits no specificity. In isolate Colombia 1, lower toxic activity was not observed for the non-selective toxic metabolites extracted with n-butanol compared to the selective compounds extracted with methylene dichloride. This observation, and the fact that both compounds were equally active on a dry weight basis, suggest again that different compounds and not analogs, or degradation products are being produced by isolate Colombia 1.

With numerous toxins, specific biological activity is sufficiently high so as to presume homogeniety (5). For example, effects of the Alternaria mali toxin (AM-toxin) can be seen at concentrations of 0.1 ng/ml, A. kikuchiana (AK-toxin) at 1-10 ng/ml, Helminthosporium maydis race T toxin (HM T-toxin) at 5-8 ng/ml, H. victoriae toxin (HV-toxin) at 0.2 ng/ml, H. sacchari toxin (HS-toxin) at 10 ng/ml, and Periconia circinata toxin (PC-toxin) at 1 ng/ml (5). On the other hand, a recently purified and characterized host-specific toxin produced by Alternaria brassicae had the lowest biological activity on the most susceptible host at a concentration 30 ug/ml. Other hosts developed symptoms only with 120 to 240 ug/ml (1).

Biological activity per unit weight of the toxic compounds produced by P. griseola and eluted from a C_{18} column were comparable to the activity of the A. brassicae toxin by using a

similar leaf bioassay technique.

Toxic compounds produced by isolate Colombia 1 were further purified through flash chromatography . Compounds with selectivity towards cultivar BAT 1647 as well as those non-selective compounds with activity to both Montcalm and BAT 1647 were detected on individual elution fractions. TLC plates were used to chromatograph each toxic fraction and exposure to UV light revealed spots of different rf values which were associated with the different specificity observed; however, toxin was not recovered from the spots.

The presence of a toxic fraction with selectivity towards the cultivar BAT 1647 with rf: 0.69 on TLC plates raises new questions on the possible role for all of the toxic compounds detected. This last toxic compound detected may be an important determinant of disease for P. griseola while the non-selective compounds which eluted together from a C_{18} column may not. Unfortunately, attempts to determine the biological activity per unit weight of each fraction was not possible. Dilution end point studies of these toxic fractions could not be conducted due to the high lability of the toxic compounds at this stage of purification.

For isolate Michigan 5, several contaminants were detected as large non-toxic peaks when the 50% ethanol eluate from a C_{18} column was run on HPLC. In addition, three different compounds were separated by HPLC with selectivity towards the susceptible cultivar Montcalm. Specific activities for these peaks were not

determined because direct dry weights could not be determined. Almost 100% of the sample weight injected into the HPLC was eluted as non toxic peaks suggesting that the toxic activity actually detected probably had much higher biological activity than the apparent activity. This also suggests that toxin is probably produced in low amounts in the culture filtrates.

HPLC of toxic compounds produced by isolate Colombia 1 showed an elution pattern with more peaks than that for isolate Michigan 5. Several peaks differing in selectivity towards the two cultivars were found. Peaks with selective toxicity towards cultivar BAT 1647 were again detected through HPLC. At least two peaks eluted very close to each other with retention times of 20 and 21 minutes. Two non-selective peaks were also detected with retention times of 28 and 29 minutes. Finally, a peak with selectivity towards cultivar Montcalm with a retention time of 33 minutes was also detected.

The use of several chromatographic techniques, particularly HPLC and flash chromatography on silica were useful in detecting and partially separating toxic compounds produced by P. griseola. The techniques also demonstrated the presence of impurities in the samples, suggesting that additional work is needed to obtain cleaner preparations. Flash chromatography seems to work much better than chromatography on C_{18} columns. It might be possible that toxic fractions eluted from flash chromatography give less contaminants when run through HPLC on silica columns too. The use of different solvents for elution on flash columns as well as on

HPLC might also reduce contaminants and result in a purer toxic preparation.

Instability of preparations appeared directly related to increased purification. This problem was partially overcome by storing samples at low pH (approximately 3.5) and adjusting to near 7.0 before leaf assays were conducted. Certainly knowing what the toxic substances are would make it possible to design treatments to neutralize them during purification and subsequent storage (3).

The choice of cultural conditions for pathogen growth is an important factor affecting toxin production (20). In order to optimize toxin yields, specific studies on nutrition such as requirements for carbon, nitrogen, inorganic ions, oxygen, and growth factors are needed (20).

Environmental factors such as temperature, pH, and light also influence toxin production and may have been responsible for the low yields. P. griseola isolates exhibited relatively good growth on Fries medium supplemented with yeast extract. However, this pathogen produced lower levels of toxic substances than other toxin producing fungi on the same artificial medium (14,20). Additional studies are needed to define nutritional conditions which are optimun for the production of toxic substances by the ALS pathogen.

Development of purification protocols require bioassays that are highly sensitive and quantitative (24). The leaf puncture bioassay used in these studies is presumably less sensitive than

other assays based on previous reports (24). A more sensitive assay is needed for the study of the toxic metabolites produced by P. griseola. For example, protoplasts were 900 times more sensitive to T-toxin than a leaf puncture bioassay.

Fairly sensitive bioassays such as electrolyte leakage and inhibition of root growth did not yield consistent results in these studies, and were therefore discarded.

Definitive evidence that toxic compounds are involved in ALS symptomatology is still lacking. Specificity of the toxic compounds isolated from culture filtrates was observed; however, there was only a 10 fold difference in the concentration needed to induce the same response in susceptible and resistant cultivars. Additional resistant and susceptible cultivars should be tested to confirm these differences.

The toxic compounds isolated in this study were of low biological activity, and it is not clear as to their significance in disease. There are no known non-pathogenic isolates in P. griseola with which to confirm the association of toxin production and disease. Moreover, P. griseola has no known sexual stage which would permit genetic studies of toxin production; methods for asexual reproduction are not available. Production of mutants would seem a logical alternative to determine the role of these toxic compounds in the disease process.

Cleaner preparations are needed for more specific studies. It would be very interesting to find out if pathotypes of P. griseola produce different compounds with selectivity to

different hosts. Both Helminthosporium carbonum and H. maydis exhibit two races. However, one race is not known to produce toxin while the other does. Alternaria alternata does not exhibit races but pathotypes which are separated by the different hosts to which they are pathogenic. Each pathotype produces a different toxin which is specific for its particular host. Strains of A. alternata were found to produce two different toxins (AK- and AM-toxins) in culture; they were pathogenic to both hosts (Japanese pear and apple leaves) (8).

In summary, P. griseola was found to produce in vitro several toxic compounds that induced symptoms in susceptible bean cultivars similar to those of ALS. The toxic compounds appeared to be different depending on pathogen isolate. A less pathogenic isolate (Michigan 5) yielded toxic compounds which were 10 times more active on the susceptible bean cultivar Montcalm than on the resistant cultivar BAT 1647. On the other hand, an isolate (Colombia 1) pathogenic on both cultivars yielded toxic compounds which were equally active on the same two cultivars.

Toxic compounds produced by isolate Michigan 5 were extracted from culture filtrates with methylene dichloride. Toxic compounds produced by isolate Colombia 1 were of two kinds. One was soluble in methylene dichloride and toxic only to Montcalm, and the other was soluble in n-butanol and toxic to both cultivars. When the n-butanol extracts of Colombia 1 were passed through HPLC or chromatographed on a silica column, fractions were obtained which exhibited toxicity to one or both of the

cultivars.

More active preparations (on a weight basis) were obtained from the filtrates by gel filtration on Sephadex, adsorption on C_{18} or silica, and HPLC. However, the most active preparation was shown by HPLC to contain non-toxic contaminants, indicating that additional work on purification procedures are needed. The best preparations, however, were active in the ug/ml range and possible at the ng level. A possible role for these toxic compounds in the ALS disease is suggested by the different activity and selectivity found in some toxic fractions. However, more experiments are needed to confirm the results obtained in these studies.

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CHAPTER III

ISOENZYME VARIATION IN ISOLATES OF *Phaeoisariopsis griseola*

INTRODUCTION AND LITERATURE REVIEW

Angular leaf spot of beans (*Phaseolus vulgaris*) caused by *Phaeoisariopsis griseola* is an economically important disease which has not been extensively studied. Several pathotypes of this fungus are described in the first chapter of these studies; pathotypes are defined on the ability or inability of an isolate to cause disease on a series of host differentials. However, the establishment of pathotypes on this basis is subject to variation in inoculation techniques, environmental growth conditions and the subjectivity of scoring for disease (7).

Several other methods to classify species or races of plant pathogens have been suggested (7), and include 1) growth characteristics and morphology on various media; 2) inhibitory effects of antifungal compounds; 3) production of a specific toxin; 4) DNA differences; 5) variation in isozyme patterns; and 6) serological methods.

Fungal taxonomy has been based largely on morphological characters, especially asexual and sexual reproductive structures. However, identification of species or genera is often made difficult by the absence of reproductive structures and by questionable reliability of taxonomic criteria based on some

features of these structures.

In recent years, there has been increasing interest in new approaches to classification of fungi at several taxonomic levels using biochemistry, serology, molecular biology, genetics and cytology (2,8). Electrophoretic patterns of soluble enzymes and other proteins in the fungal cell are some direct manifestations of the cell's genome and those patterns have been of great value in fungal taxonomy (5,8).

Starch and polyacrylamide gel electrophoresis have been applied to several taxonomic problems. For protein banding patterns, there is a large number of proteins present in crude extracts which are sometimes difficult to separate in one gel; however staining of particular enzymes simplifies the patterns (2). There seems little doubt that fungal species exhibit characteristic protein patterns. Clare et al (5) indicated that enzyme patterns may show a high degree of intraspecific variability and concluded that enzyme patterns are of greater value than total protein patterns in characterizing subspecific taxonomic groups.

The genus Phytophthora, which includes a large number of economically important plant pathogens, is an example of a group of plant pathogens in which species are difficult to identify. Gel electrophoresis has been very useful in separating Phytophthora species. For example, soluble proteins from mycelia of 17 isolates belonging to seven Phytophthora spp. and varieties were separated into seven distinct types of protein patterns.

corresponding to the seven species and varieties (26). Similar results with Phytophthora were reported by other researchers (5,6,11) who indicated that protein and enzyme patterns among species are different; however, some bands may be common to the different species.

Hall et al (11) examined the electrophoretic patterns of buffer soluble proteins from 43 isolates of Phytophthora and separated them into the three species: P. cinnamonni, P. cactorum, or P. palmivora. Isolates of P. cinnamonni produced essentially identical protein patterns, but 3 different esterase patterns. Among isolates of P. cactorum, one protein pattern and one esterase pattern were recognized. For P. palmivora isolates, two protein patterns and nine esterase patterns were identified. They indicated that protein patterns within a species were more similar than patterns from other species and pointed out the variability often enccuntered within species of this genus in the esterase patterns. They did not indicate if the variability of the esterase patterns within a species was associated with races of the pathogen.

Protein patterns in most Phytophthora species are identical or nearly identical regardless of the date of isolation, host from which isolated, or geographic locality (6,11,12,26).

Lack of association between pathogencity and electrophoretic protein patterns of all isolates in the eight races of P. fragariae was observed by Gill and Powell (10). On the other hand, within P. megasperma, several groups of isolates were

distinguished by their protein banding patterns; isolates from related hosts were generally similar (12,14).

Irwin and Dale (14) consider that the "forma specialis" grouping previously established for soybean and lucerne isolates of P. megasperma should be preserved. Their results showed that protein profiles were identical for the nine isolates from chickpea or lucerne, but differed significantly for isolates of P. megasperma f.sp. glycinea, P. vignae, and P. nicotianae var. parasitica. In a separate study, Hansen et al (12) compared electrophoretic patterns of soluble proteins of 93 isolates of the same fungus pathogenic on alfalfa, soybean, clover, douglas fir, rosaceous fruit trees, and a broad range of hosts. Electrophoretic patterns were highly correlated with the hosts from which isolates were obtained.

Electrophoretic patterns of soluble proteins and enzymes have also been used to classify other fungal species. Among the fungi examined were Sclerotinia minor, S. sclerotiorum, and S. trifoliorum (8,18,22,25); Pythium spp (5); and Rhizoctonia- like fungi (21). Pectic zymograms of 140 Rhizoctonia isolates were used to separate the isolates into 11 groups. Each group was distinguished on the basis of type of lesions and virulence towards their hosts.

Electrophoretic patterns of sporangiospore proteins from Rhizopus spp. contradicted the current classification of Rhizopus species, lending support for a reduction in the number of species (19). When electrophoretic patterns of 30 Botrytis isolates were

examined for total proteins, arylesterase, acid and alkaline phosphatases, and glucose-6-phosphate dehydrogenase, six different clusters were identified. Each cluster corresponded to one of the six species: B. cinerea, B. aclada, B. fabae, B. gladiorum, B. tulipae, and B. viciae. Most differences were due to arylesterase patterns, and isolates could not be correlated with geographical origin, or sclerotial and conidial production (1).

Enzyme polymorphism of esterases, aminopeptidases, and phosphatases can be used to distinguish the three species Pleurotus eryngii, P. ferulae, and P. nebrodensis corroborating differences in morphology (2).

Gel electrophoresis of soluble proteins and enzymes has been used to differentiate races of fungal plant pathogens. Gill and Powell (10) were unable to separate eight physiological races of Phytophthora fragariae on the basis of proteins in spore extracts. Electrophoretic patterns of the proteins were evaluated on the basis of number, position, and density of the bands present.

Physiological races of Phytophthora infestans produced essentially the same protein patterns. There were some differences in the electrophoretic patterns for the different isolates, but the authors could not associate the differences with different races (26).

Protein patterns obtained with spore extracts from physiological races of Puccinia coronata f.sp. avenae revealed

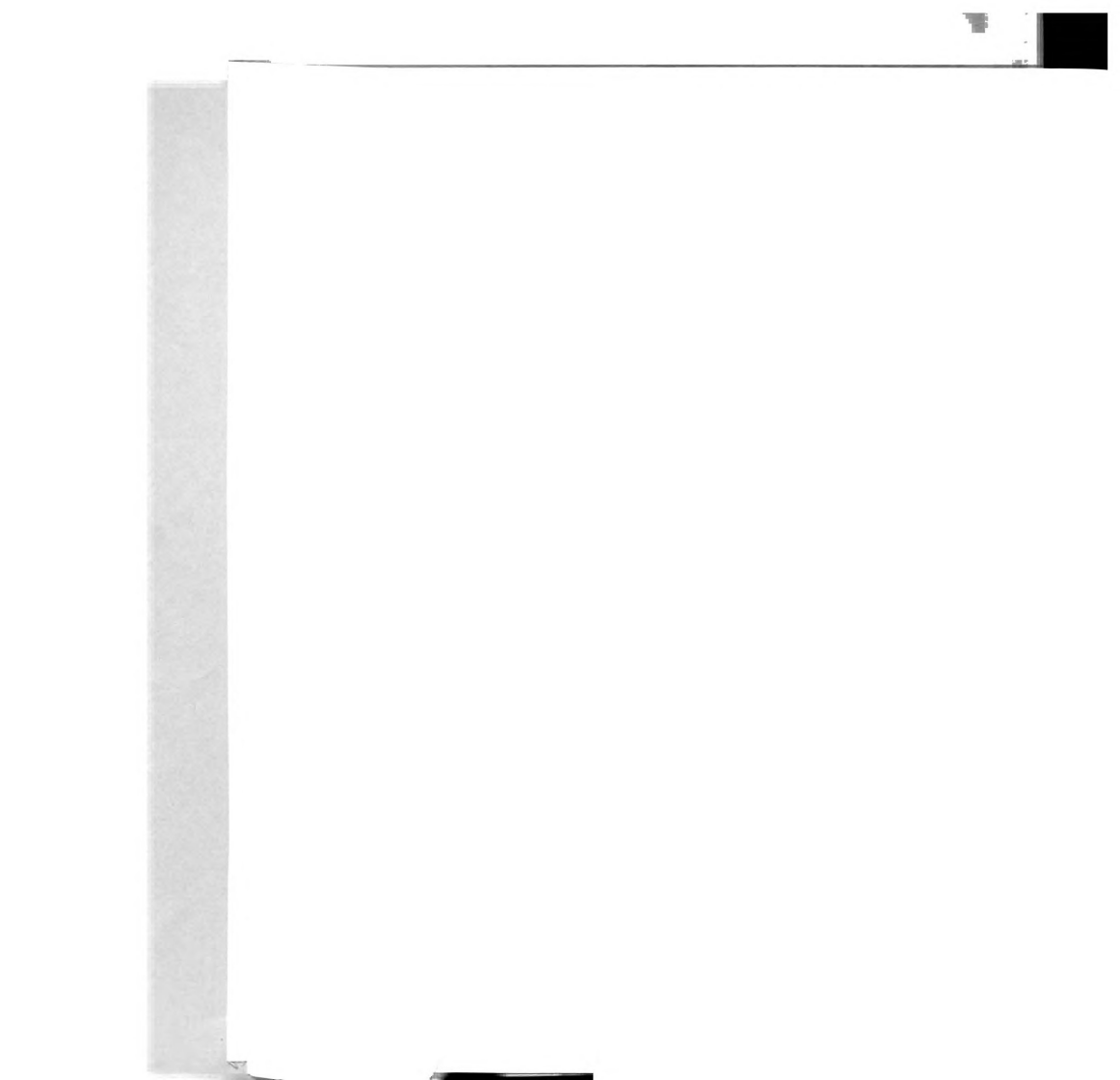
no differences (20). In addition, Burdon et al (3) examined isozymes in 58 isolates representing seven pathotypes of Puccinia graminis f.sp. tritici and 66 isolates representing six pathotypes of P. recondita f.sp. tritici. No variation in isozyme phenotypes of 11 different enzyme systems was noted for both pathogens.

No correlation was found between two races among 26 isolates of Rhynchosporium secalis and the enzyme patterns for esterase, peroxidase, acid and alkaline phosphatase, B-glucosidase, and B-galactosidase enzyme systems (Newman, P.L. 1978, in Newman, P.L.1985 (16)). In the same report, large isozymic variation was found in mycelial extracts of 288 single spore isolates of R. secalis, the causal agent of leaf blotch of barley. A total of 28 patterns for esterase, four for B-glucosidase, and one for B-galactosidase were found. Newman found one probable isozyme polymorphism for esterase; there was no correlation between any particular band or pattern and either host-cultivar or origin of isolate (16).

Inspite of all of these negative reports, Macko et al (15) claimed to have differentiated races 21 and 111 of Puccinia graminis var tritici by PAGE of proteins and indicated that race distinctions may be possible in other fungal species by use of the same technique.

In this chapter, studies were conducted to determine the amount of isozymic variation present in populations of Latin American and African isolates of P. griseola. Variation was

sought to try to place isolates into related groups and to determine the association between isozymic patterns and the pathotypes reported in the first chapter of these studies. In addition, variation was sought to provide a natural marker system that could be used in further studies on the nature of pathogenic variation within the ALS pathogen.



MATERIALS AND METHODS

Isolates and Growth Conditions

The P. griseola isolates used in this study are listed in Table 21. Isolates were maintained on V-8 juice agar plates as described previously. Mycelium for electrophoresis was obtained from 25 ml of liquid culture of modified Fries medium (see chapter two) or Czapek-Dox-solution ($NaNO_3$, 2.0 g; KH_2PO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KCl. 0.5 g; $FeSO_4.7H_2O$, 0.01 g; sucrose, 30 g; H_2O to 1 liter).

Flasks (125 ml) containing 25 ml of media were inoculated with a small plug of mycelium plus agar from cultures grown for 10-12 days on V-8 juice agar. Liquid cultures were incubated for several days as indicated in the results either under stationary conditions or on a rotary shaker. Mycelium was harvested by vacuum filtration, dried in a piece of paper toweling, and either stored at -20 C or used immediately for extraction of enzymes.

Extraction of Samples

Dried mycelium (0.5 to 0.6 g per sample) was ground with a small mortar and pestle in one ml of extraction buffer containing a small amount of sand. The extraction buffer (16) contained 170 g sucrose, 1 g ascorbic acid, and 1 g cysteine hydrochloride in one liter of tris-citrate buffer, pH 8.7. The resultant slurry was transferred to a 9 ml centrifuge tube. The macerated sample

was centrifuged at 20,000 or 40,000 g at 4 C for 45 min. The resulting supernatant was absorbed onto paper chromatography wicks (5x12 mm^2). Loaded wicks were either used immediately or kept in the freezer until needed.

Electrophoresis

For electrophoretic analyses, wicks containing the samples were inserted into a single sample slot in a horizontal starch gel (12% W/V). Each sample was replicated three times, and gels were run with a discontinuous buffer system. A constant current of 45 mA was maintained for those enzyme systems developed in borate or citrate buffer systems. The voltage was kept constant at 300 V for histidine buffer systems. In all three systems, samples were run until the bromphenol blue tracker dye had migrated 9 cm from the sample slot. All samples were pre-electrophoresed for 10 min to unload the samples from the wicks into the gel; the wicks were then removed and electrophoresis continued.

Each gel was cut horizontally into three or four slices and assayed for enzymes. On citrate gels, enzymes assayed were esterase (EST), catalase (CAT), leucine aminopeptidase (LAP), diaphorase (DIAP), methylumbelliferyl-esterase (MU-est), methylumbelliferyl-galactosidase (MU-B gal), methylumbelliferyl-glucosidase (MU-B glu), peptidase (PEP), and fumarase (FUM). On histidine gels, enzymes assayed were malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6PDH), glutamate

dehydrogenase (GDH), isocitrate dehydrogenase (IDH), 6-phosphogluconate dehydrogenase (6PGDH), adenylate kinase (AdK), fructose kinase (FK), and phosphoglucomutase (PGM). On borate gels, enzymes assayed were glutamate oxalate transaminase (GOT), peroxidase (PRX), shikimate dehydrogenase (SKDH), glucose phosphate isomerase (PGI), alcohol dehydrogenase (ADH), acid phosphatase (ACP), malic enzyme (ME), glucose-1-phosphate transferase (G1PT), and hexokinase (HK). Enzyme activity on the gels were detected using previously described techniques (13,24).

For each isolate, a minimum of three replicates per gel and three samples from separate cultures were run on different gels.

The effects of culture (Fries vs Czapek) and incubation conditions (stationary vs shaken) on the elctrophoretic patterns of five enzymes (EST, CAT, LAP, PRX, ACP) were studied with eight African and three Latin American isolates.

The 26 enzyme systems described above were analyzed for 14 different P. griseola isolates to select the enzymes exhibiting isozymic variation. The isolates included were: 8 African isolates (Rwanda 1 and 3; Malawi_4 and 5; Burundi 2; and Tanzania 2 and 3) and 6 Latin American isolates (Costa Rica 1; Brazil 1 and 3; Argentina 3; Colombia 4; and Nicaragua 1).

RESULTS

Preliminary Experiments

Electrophoretic patterns of 16 different enzymes extracted from mycelia of P. griseola isolate Michigan 5 grown for 7, 14, 21, 30, and 60 days on modified Fries medium were determined. The best resolution observed for the different patterns was obtained for cultures 14 to 21 days old. Most of the electrophoretic bands were also seen with cultures of 7 or 30 days age; however, these bands were much less intense, and some were absent. Electrophoretic patterns of cultures of 60 days age showed little or no activity for most of the enzymes.

Eleven different Latin American and African isolates and five enzyme systems were used to compare incubation conditions of stationary or shaking and growth on Fries or Czapek media. Electrophoretic patterns in mycelial extracts of 14 day cultures showed no differences for any of the treatment combinations. Banding patterns for each enzyme were identical in number, position, and intensity regardless of media or incubation conditions. However, differences in banding patterns of a particular enzyme were observed between isolates as will be dicussed later. In all subsequent studies, mycelial extracts were obtained from cultures after 14 days stationary growth in Fries medium.

Four of 26 enzymes assayed showed pattern variation among 14

isolates when mycelial extracts were evaluated. These enzymes were: esterase (EST), catalase (CAT), leucine aminopeptidase (LAP), and adenylate kinase (AdK). These four enzymes were then selected for subsequent studies.

The remaining enzyme systems were not studied further either because there was no variability among isolates or because enzyme activity was not detected. For example, all isolates exhibited two common bands for malate dehydrogenase (MDH), and one common band for acid phosphatase (ACP), methyumbelliferyl-B galactosidase (MU-B gal), methyumbelliferyl-B glucosidase (MU-B glu), and peroxidase (PRX). Moreover, the intensity of the bands varied between experiments making their use unreliable.

The enzyme methyumbelliferyl-esterase (MU-esterase) showed an electrophoretic pattern identical to EST. The enzymes isocitrate dehydrogenase (IDH), and fumarase (FUM) were never detected in the assays.

Resolution of isoenzymes of the remaining 14 enzyme systems described in materials and methods was a problem in starch gels. Resolution was poor and zones of enzyme activity were diffuse, often up to a distance of three centimeters.

Isoenzyme variation among 55 P. griseola isolates.

The EST, LAP, CAT, and AdK isozyme patterns in 55 pathogen isolates revealed major differences; the results are summarized in Figure 6. Eight EST, three CAT, two LAP and two AdK bands were identified. All isolates were separated into two patterns.

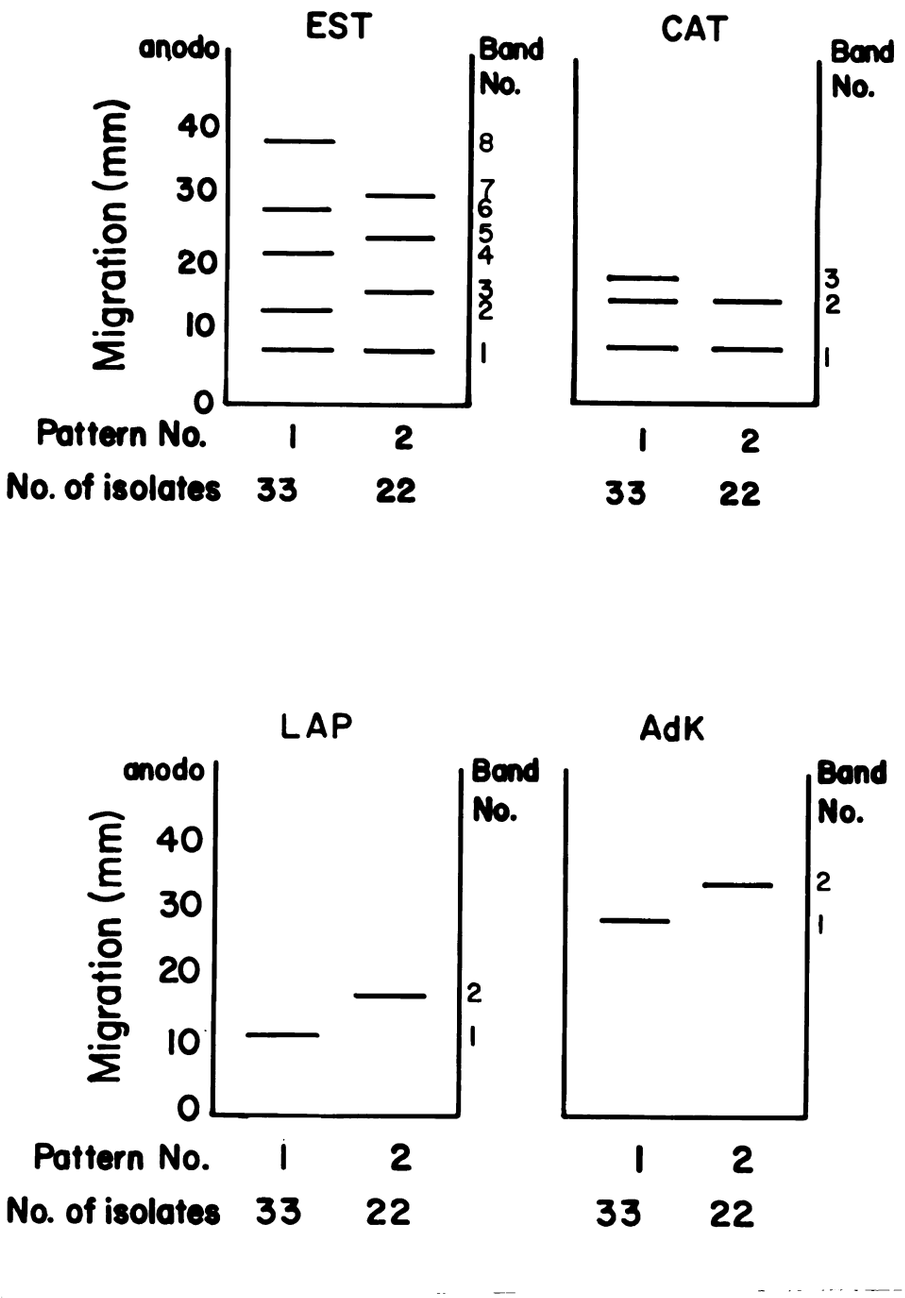


Figure 6. Esterase (EST), catalase (CAT), leucine aminopeptidase (LAP), and adenylate kinase (AdK) patterns distributed among 55 isolates of P. griseola. Isoenzymes were separated by using starch gel electrophoresis of mycelial extracts obtained from isolates grown in Fries medium in stationary conditions for 14 days.

Pattern one for each enzyme was exhibited by the same 33 isolates indicated in Figure 6 and Table 21, while pattern two was exhibited by the same 22 isolates as indicated.

The electrophoretic patterns found in all 55 of the P. griseola isolates are summarized (Table 21) on a country basis. Pattern 1 for the four enzymes was exhibited by all 26 African isolates, six Latin American isolates, and one Michigan isolate. On the other hand, pattern 2 contained 22 isolates, all from Latin America. In general, it would appear that isozymic variation in P. griseola is associated with origin of the isolates, African isolates representing electrophoretic pattern 1 and Latin American isolates representing pattern 2.

There were differences among 38 isolates of P. griseola relative to pathotype, origin of isolates, and electrophoretic patterns of the four enzyme systems as summarized in Table 22. Data presented in the table do not reveal any useful association between electrophoretic patterns and pathotype or race. Although the pathotypes differ greatly in pathogenicity, only two electrophoretic patterns were found. The most pathogenic pathotypes which are in the upper part of Table 22 exhibit either electrophoretic pattern 1 or pattern 2. On the other hand, the pathotypes showing the lowest levels of pathogenicity exhibited only electrophoretic pattern 1, regardless of origin.

Pathotypes 1 and 9 were the only ones containing isolates exhibiting different electrophoretic patterns; the remaining 12 pathotypes contained isolates exhibiting either pattern 1 or

Table 21. Electrophoretic patterns for four enzymes found in 55 isolates of P. griseola from Latin America, Africa, and USA*.

Origin of Isolates	Isolates	Electrophoretic Patterns for EST,CAT,LAP,AdK
Latin America		
Colombia	Col 1,2,5,6	2
	Col 4,7,8,9	1
Brasil	Br 1,2,3,5,6,7	2
Argentina	Arg 1	2
	Arg 3	1
Guatemala	Guat 1,2,3,4,5	2
Costa Rica	CR 1,2	2
Nicaragua	Nic 1	1
Mexico	Mex 1	2
Puerto Rico	PR 2,4	2
Dominican Republic	DR 1	2
Africa		
Malawi	Mal 2,4,5,7,9,10,11	1
Tanzania	Tz 1,2,3,4,5	1
Kenya	Ken 1,2,3	1
Uganda	Ug 1,2,3	1
Burundi	Bu 1,2,3	1
Rwanda	Rw 1,2,3	1
Zaire	Za 1,2	1
USA (Michigan)	Mich 5	1

*Electrophoretic patterns for esterase (EST), catalase (CAT), leucine aminopeptidase (LAP), and adenylatekinase (AdK).

Table 22. Relationship between pathotype, origin of isolate, and electrophoretic pattern of four enzymes in 38 *Phaeoisariopsis griseola* isolates.

Pathotype[a]	No. Isolates[b]	Origin of Isolates[c]			Electrophoretic Pattern[d]	
		A	LA	USA	1	2
1	3	1	2		1	2
2	3	3			3	
3	2		2			2
4	1	1			1	
5	3		3			3
6	2	2			2	
7	7	7			7	
8	1		1			1
9	2	1	1		1	1
10	1		1			1
11	2	2			2	
12	2	2			2	
13	1		1		1	
14	8	5	2	1	8	
No. iolates	38	24	13	1	28	10

[a]Pathotypes determined on a set of eight bean diiferentials. Pathotypes are in decreasing order of pathogenicity where pathotypes 1 and 14 are the most and least pathogenic respectively.

[b]Number of isolates with known pathotype used for electrophoresis

[c]A=number of African isolates; LA=number of Latin American isolates; USA= number of USA isolates

[d]Electrophoretic patterns 1 or 2 determined by starch gel electrophoresis for esterase, catalase, leucine aminopeptidase, and adenylate kinase.

pattern 2.

Table 23 shows the four different enzyme systems studied with the assigned band numbers (Figure 6) and Rf values for each band. Two enzymes systems (EST and CAT) were considered to be polygenic. EST is suggested to be probably polymorphic for EST bands 6 and 7; 4 and 5; and 2 and 3 respectively in Figure 6. LAP (Table 23, Figure 7) and AdK (Table 23, Figure 8) were considered polymorphic at one locus.

Table 23. Band number and Rf values for EST, CAT, LAP, and AdK enzyme systems in Phaeoisariopsis griseola[a]

Isoenzyme System	Band No[b] Pattern 1	Band No[b] Pattern 2	Rf[c]
EST	1	1	0.09
	2		0.16
		3	0.18
	4		0.24
		5	0.27
	6		0.31
		7	0.33
	8		0.42
CAT	1	1	0.09
	2	2	0.17
	3		0.20
LAP	1		0.13
		2	0.19
AdK	1		0.31
		2	0.37

[a]Enzyme systems studied were esterase (EST), catalase (CAT), leucine aminopeptidase (LAP), and adenylate kinase (AdK). Fifty-five isolates from 14 different pathotypes were used for starch gel electrophoresis.
[b]Assigned band numbers for each pattern (Figure 6).
[c]Rf= distance migrated by a band from sample slot/distance migrated by the tracker dye.

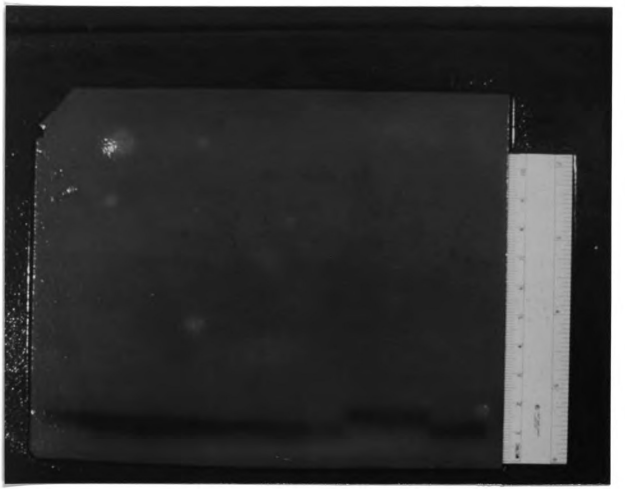


Figure 7. Leucine aminopeptidase (LAP) isoenzyme pattern 1 (Rf= 0.13) and pattern 2 (Rf= 0.19) in 10 P. griseola isolates. From left to right (3 replications/isolate): Malawi 9, Rwanda 2, Malawi 11, Malawi 2, Uganda 3, Colombia 8, Kenya 2, Brazil 6, Colombia 6, and Tanzania 5. Samples obtained from mycelial extracts were electrophoresed on citrate starch gels at a constant current of 45 mA until the tracker dye migrated 9 cm from the sample slot.

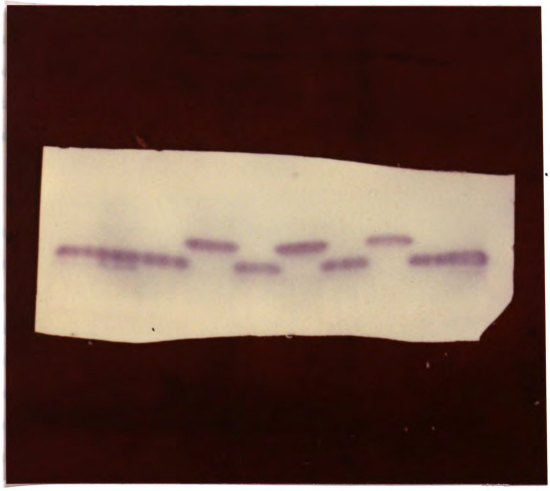


Figure 8. Adenylate kinase (AdK) isoenzyme pattern 1 (Rf= 0.31) and pattern 2 (Rf= 0.37) in 10 isolates of P. griseola. From left to right (3 replications/isolate): Zaire 1, Nicaragua 1, Uganda 2, Brazil 1, Michigan 5, Guatemala 5, Tanzania 2, Puerto Rico 2, Tanzania 4, and Argentina 3. Samples obtained from mycelial extracts were electrophoresed on histidine starch gels at a constant voltage of 300 V until the tracker dye migrated 9 cm from the sample slot.

DISCUSSION

Investigations of the electrophoretic patterns of soluble enzyme extracts obtained from 55 P. griseola isolates showed clear variation in the number and position of bands for the four enzyme systems EST, CAT, LAP, and AdK. These results suggested that starch gel electrophoresis of the four enzymes could be used to separate isolates of P. griseola into two different groups.

Variability in patterns produced by a single isolate in successive experiments was examined. Results indicated that variability existed in that not all bands were equally well-resolved. However, a characteristic pattern of band number and position was always observed. This variability was reduced when the extracts were prepared from cultures 14 to 21 days old. On the other hand, the number of bands, position, or intensity of the bands was not influenced by the cultural conditions of growth.

Physiologic race differences can be, but not always, associated with differences in electrophoretic patterns of spore protein and enzyme extracts in other pathogens (3,10,15,16,20). Such a relationship was not found for the 14 pathotypes of P. griseola.It appears that small changes in pathogen genomes which may affect pathogenicity will not always be associated with electrophoretic patterns obtained from mycelial extracts. It is possible that the technique used here was not sufficiently

sensitive to detect all minor differences in enzyme systems.

For two races of Fusarium oxysporum f.sp. pisi, Coddington et al (7) correlated pathotypes as defined by pathogenicity tests on a series of host differentials with DNA structure, as defined by restriction digestion analyses. The authors recommended classification of isolates into races on the basis of their restriction enzyme digestion patterns as an improved, more reliable and reproducible method for race classification.

In the present study, just two isozymic patterns for each enzyme system were detected in the 14 pathotype groupings (Tables 21 and 22), established for the 55 isolates of P. griseola.

A genetic interpretation of the different bands showed in Figure 6 for each enzyme system is not possible since genetic studies were not carried out.

According to Figure 6, EST band No 8 was present in pattern 1 but absent in isolates with pattern 2, which may be the result of an allele coding for an inactive form of the allozyme (16,17), or the result of a reduction in the level of activity of the enzyme in pattern two that was not detected by the technique used here. EST bands 2 and 3; 4 and 5; and 6 and 7 are possibly allozymes of several isoenzymes that seemed to exhibit polymorphism. These allozymes of similar molecular weight (similar Rf) would be under different allelic control assuming a single locus for each pair of bands (17). Polymorphic isoenzymes are allozymes coded for by different alleles at a single locus.

This kind of genetic polymorphism is normally described as fast and slow variants of an isoenzyme with similar tissue activities (17). Different isoenzymes on the other hand, have different molecular weights and are active in different host tissues. EST band No 1 was a common band in both pattern 1 and pattern 2 and could be considered to be the same allozyme. EST was the most complex system found among all of the enzymes detected in P. griseola.

The CAT enzyme system exhibited 3 electrophoretic bands (Figure 6). Both CAT bands No 1 and 2 were present in both electrophoretic pattern 1 and pattern 2 and could be considered that each, band No 1 as well as band No 2 in each pattern are the same allozymes. CAT band No 3 was prresent in pattern 1 but absent in pattern 2.

The two enzyme systems (LAP and AdK) might exhibit polymorphism at a one locus, with the fast variant present in pattern two and the slow variant in pattern 1 for both systems (Figure 6). Considering no polymorphism for the CAT system, all fast variants for the three enzyme systems (EST, LAP, and AdK) would be present in pattern 2 while all the slow variants would be present in pattern 1.

Unfortunately, it is not possible to test polymorphism by genetic analyses since P. griseola has no known perfect stage, and alternate methods of reproduction are not known which would permit genetic recombination.

Isoenzyme phenotypes for eight enzymes were used to assess

the origin and evolution of Puccinia graminis f.sp. tritici in Australia (4). The authors postulated that most of the major changes in the wheat stem rust pathogen population of Australia have resulted from overseas introductions of the pathogen. In particular, the uniformity of isoenzyme phenotypes of the Australian rust pathogen population seemed to be largely explained on the basis of a common origin for all existing pathotypes (3).

The origin of the two different isozyme phenotypes observed for the P. griseola population studied is open to speculation. Interestingly, there was a correlation between origin of the isolates and their electrophoretic pattern for the four enzyme systems.

There were 26 African isolates of the pathogen, from seven different countries, and all exhibited electrophoretic pattern number one. Pattern one was also found in six Latin American isolates and one isolate from the USA. Twenty-two of the 28 isolates from 13 Latin American countries exhibited pattern 2. The number of P. griseola isolates tested is representative of different geographical areas within each continent. These results thus suggest that electrophoretic pattern one originated in the African continent. The six isolates with the same pattern found in 3 Latin American countries plus the isolate found in USA could have been introductions from Africa. The results also suggest that electrophoretic pattern two originated in Latin America, and that it probably has not been introduced into Africa.

A different interpretation of the origin and evolution of these two electrophoretic patterns in P. griseola is possible. Origin of the common bean (Phaseolus vulgaris) is associated with some areas in Central and North America, including samples found in Mexico that date to 7,000 years ago. Origin of beans is also associated with the Andean zone in South America where samples probably 7,500 to 10,000years old have been found in Peru (9,23). It is also believed that the small seeded types of beans which are cultivated in Central America, Mexico, the Antillas, Venezuela, and Brazil all have a common origin in Central America and Mexico (9,23). On the other hand, the Andean countries seem to prefer beans of large seed types which probably became distributed in Colombia, Peru, Argentina, and Chile (9,23). This type of bean is believed to have originated in the Andean zone.

Studies of pathogenicity in P. griseola have indicated an association between the less pathogenic groups and seed type. For example, pathotype 13 is pathogenic on large seeded differentials such as Montcalm and G 5686, and pathotype 14 is pathogenic on Montcalm. These two pathotypes are non pathogenic on the 6 small seeded bean differentials. Table 22 in this chapter shows that the less pathogenic pathotypes also exhibit only electrophoretic pattern one. Unfortunately, the host cultivars from which the isolates of the fungus were obtained are not all known.

There is a preference in Latin America for small-seeded types (23). This preference, and the electrophoretic patterns

obtained for Latin American isolates suggest an association between small seeded type and electrophoretic pattern two. For example, Brazil and Guatemala produce only small seed types (23), and only electrophoretic pattern 2 was found for the 6 Brazilian and 5 Guatemalan isolates (Table 21). Costa Rica and most of the Central American countries also produce mainly small seed types and electrophoretic pattern 2 was associated.

Of the eight P. griseola isolates from Colombia, four exhibited electrophoretic pattern 1 and four pattern 2. Although Colombia has a preference for large seeded types, there is a possible explanation for the existence of patterns 1 and 2. The four isolates which exhibited electrophoretic pattern 2 were collected from the areas Santander de Quilichao, Popayan, and Restrepo in experimental fields from CIAT. Most of the beans grown in these experimental fields are of small seeded types. On the other hand, three of the isolates with pattern 1 were obtained from the areas Nariño, Antioquia, and Quindio where only large seeded types are traditionally produced. The fourth isolate was from an experimental field at Popayan where mixtures of both seed types are found, being possible that the isolate was originally collected from a large seeded type.

In the case of Argentina, where one isolate was of pattern one and another isolate of pattern two, there is also a similar explanation. Most of the bean production in this country is for export and consists of both large and small seeded types (23). Thus, it is possible that the two isolates originated from

different seed types.

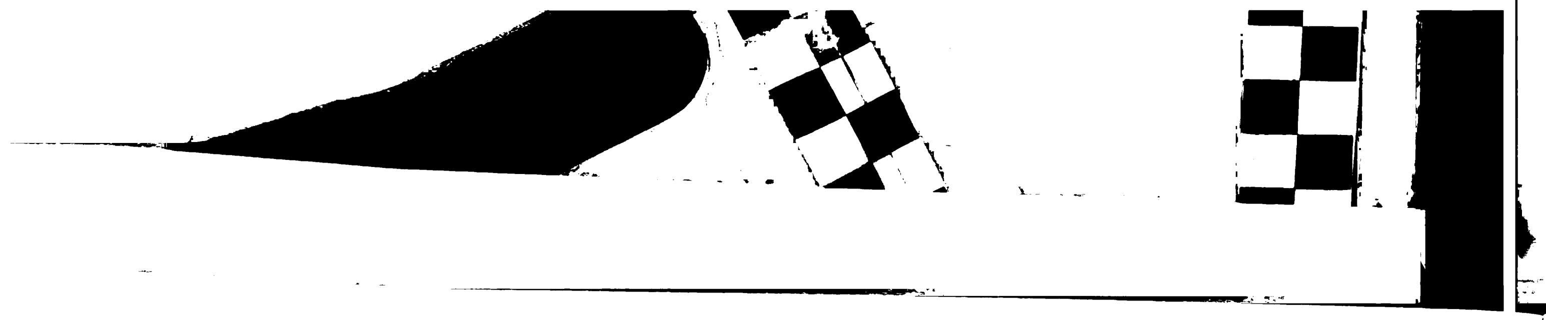
Single isolates from Mexico and from Nicaragua yielded patterns 2 and 1 respectively. However, both electrophoretic paterns might be expected in these countries as small, medium, and large seed types are grown. The remaining isolate with pattern one was from the USA and it was originally isolated from the large seeded cultivar Montcalm of Andean origin.

The fact that only 25% of the Latin American isolates yielded electrophoretic pattern 1 is probably because most of the isolates were collected from those areas were there is a larger production of small seeded bean types. Probably those isolates were originally collected from those bean types.

A similar situation may explain why electrophoretic pattern 2 was not found in African isolates. Most of the cultivated beans in the areas where isolates were collected in Africa are of large or medium seed types of Andean origin. It is possible that all samples collected for isolation of the fungus were originated from that type of beans. This suggests the possibility that isolates with electrophoretic pattern one were probably brought into Africa with the first bean introductions of large seed types.

Thus, it seems that electrophoretic pattern one did not originate in Africa and was introduced into Latin America as first suggested. The opposite seems to be the case.

It would be very interesting to now study the electrophoretic patterns of P. griseola isolates collected from a



larger population of beans with known characteristics such as color, seed size, level of infection at time of collection, location, and known environmental factors.

The great relatedness of the two electrophoretic patterns suggests that each pathogen population had a selective pressure in favor of one allele or another, probably depending on the genotypes serving as hosts. A common origin for the two electrophoretic patterns found is difficult to establish at this point. However, it is possible that one of the patterns originated first and the other pattern arose via a mutation, or pattern 1 originated in the Andes and pattern 2 in Midle America.

Gepts (9) suggested that coevolution between the common bean plant and associated organisms such as the bean rust pathogen (Uromyces phaseoli (Reben)Wint) or Rhizobium phaseoli, may have lead to specific interactions with common bean gene pools. He also suggested that the pathogen and the host may have different genes for virulence and resistance, respectively, in Middle America and in the Andes.

In summary, the enzyme systems esterase (EST), leucine aminopeptidase (LAP), catalase (CAT), and adenylate kinase (AdK) exhibited each two isozyme patterns. The electrophoretic patterns of these four enzymes separated 55 P. griseola isolates from Latin America and Africa into two groups. Electrophoretic patterns were associated with origin of isolates but not with pathogenicity. Pattern 1 was mainly found in African isolates while pattern 2 was only found in Latin American isolates.

An association between electrophoretic pattern and bean seed type (small-large) is suggested by the type of beans that are grown in those areas where isolates were obtained. Electrophoretic pattern 1 being associated with large seeded bean types and pattern 2 being associated with small seeded bean types. These observations indicate that each pattern could have coevolved with each seed type as they are believed to have originated at different locations in America. Pattern 1 probably coevolved with large bean seeded types in the Andean zone of South America, while pattern 2 probably coevolved with small bean seeded types in Central and North America.

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