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Genetic variability within *Phaeoisariopsis griseola* from Central America and its implications for resistance breeding of common bean

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The genetic and virulence variability of 112 isolates of *Phaeoisariopsis griseola*, collected from various locations in Central America, were studied using seven random amplified polymorphic DNA (RAPD) primers and 12 common-bean differential genotypes. Broad molecular diversity ($H = 0{\cdot}92$) among isolates was found using RAPD markers. Fifty pathotypes were identified on 12 differential bean genotypes, 29 of which were represented by only one isolate. Only 18 pathotypes were found in two or more countries. Pathotype 63-63 was the most virulent and caused leaf spots on all 12 common-bean differential genotypes. Comparison of virulence phenotypes and RAPD profiles to known Andean *P. griseola* isolates confirmed that all isolates belonged to the Mesoamerican group. Pairwise comparison between individual RAPD loci showed that the majority were in gametic phase linkage disequilibrium, revealing that *P. griseola* maintains a genetic structure that is consistent with asexual reproduction. The molecular and virulence diversities of *P. griseola* isolates from Central America imply that using single resistance genes to manage angular leaf spot is inadequate and stacking resistance genes may be necessary to manage the disease effectively.

Keywords: bean angular leaf spot, genetic variation, *Phaeoisariopsis griseola*, *Phaseolus vulgaris*, virulence diversity

Introduction

Angular leaf spot (ALS), caused by the fungus *Phaeoisariopsis griseola*, is one of the most widely distributed and damaging diseases of common bean (*Phaseolus vulgaris*) in tropical and subtropical countries (Correa-Victoria, 1988; Saettler, 1991; Liebenberg & Pretorius, 1997; Wortmann *et al.*, 1998). Yield losses on susceptible varieties can be as high as 80% (Schwartz *et al.*, 1981; Guzman *et al.*, 1995). Because the use of fungicides is beyond the means of most small-scale farmers, the most practical and preferred means of ALS management has been the use of resistant varieties. However, host resistance is difficult to maintain because the abundant virulence (pathotype) diversity of *P. griseola* renders varieties that are resistant in one location or year susceptible in another (Pastor-Corrales *et al.*, 1998). Thus, the use of a wide diversity of host resistance genes and knowledge of the pathogen's genetic diversity for virulence and other markers are useful tools in developing and deploying bean varieties with durable ALS resistance.

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Little is known about the extent of pathotype variability for *P. griseola* in Central America. Characterizing the *P. griseola* population from this region using both molecular and virulence markers will provide information on the amount of genetic variation that is maintained in this pathogen and the geographical distribution of the different pathotypes. Comparison of pathotype structure and molecular diversity may provide insights into the speed at which new virulent pathotypes will occur. In addition, comparative studies between diversity in virulence and neutral molecular markers may provide a rational hypothesis about the mechanism of pathogen variation and provide information on evolutionary factors generating and maintaining genetic diversity in the ALS pathogen population (Milgroom & Fry, 1997).

Previous studies have revealed high levels of pathotypic variation in *P. griseola* (Correa-Victoria, 1988; Guzman *et al.*, 1995; Maya *et al.*, 1995; Pastor-Corrales & Jara, 1995; Boshoff *et al.*, 1996; Chacón *et al.*, 1997; Pastor-Corrales *et al.*, 1998). For example, Marin-Villegas (1959) identified 13 pathotypes among 33 isolates from Colombia, while with Brazilian isolates, Paula & Pastor-Corrales (1996) identified 21 pathotypes among 27 isolates and Aparício (1998) identified 30 pathotypes among 66 isolates. Despite the high pathotype diversity, all *P. griseola* pathotypes have frequently been

divided into Andean and Mesoamerican groups that correspond to the two common-bean gene pools (Singh *et al.*, 1991; Pastor-Corrales & Jara, 1995; Boshoff *et al.*, 1996; Chacón *et al.*, 1997; Pastor-Corrales *et al.*, 1998). The Andean group consists of *P. griseola* isolates recovered from large-seeded common-bean genotypes of Andean origin that infect Andean genotypes only; the Mesoamerican group contains isolates that are more virulent on Mesoamerican bean genotypes (CIAT, 1995; Pastor-Corrales & Jara, 1995; Pastor-Corrales *et al.*, 1998).

A practical consequence of *P. griseola*/common-bean coevolution is that pyramiding of resistance genes from Andean and Mesoamerican gene pools might lead to effective and durable disease management. Such a strategy requires a thorough understanding of the population structure of the pathogen in the region in question. However, there is limited information on the population structure of *P. griseola* from Central America, one of the centres of common-bean domestication. In addition, characterizing the virulence of *P. griseola* populations to bean genotypes will provide important information that can be used to identify broadly effective resistance genes, develop effective resistance in varieties destined for production in this region, and deploy resistant varieties in ways that prolong their durability.

The work reported here was conducted to investigate the variation in populations of *P. griseola* in Central America using differential bean genotypes and molecular markers inferred from the random amplified polymorphic DNA (RAPD) technique. The objectives of this study were: (i) to evaluate the virulence variability and geographical distribution of *P. griseola* pathotypes in Central America; and (ii) to assess the relationship between molecular diversity and virulence variability, in order to contribute to the development and deployment of durable resistance to *P. griseola*.

Materials and methods

Fungal isolation, purification and inoculum production

One hundred and twelve single-spore isolates of *P. griseola* from Central America were used in this study (Table 1). The isolates were collected from infected bean leaves from commercial or smallholder farms between 1997 and 1999. An additional 25 isolates (six from Ecuador and 19 from Colombia) were included to elucidate the relationship between *P. griseola* isolates collected from Andean bean genotypes and those from Mesoamerican genotypes. Pathogen isolation, monosporic isolate production and inoculum preparation were performed as described previously (Pastor-Corrales *et al.*, 1998). Isolates were maintained on V8 juice agar medium at 4°C (short-term), or lyophilized and stored at 4°C for long-term storage.

Determination of virulence phenotypes of *P. griseola*

To determine the virulence phenotype and pathotype of each *P. griseola* monosporic isolate, a set of differential genotypes consisting of six Mesoamerican and six Andean bean lines (identified during the first ALS workshop held at CIAT, 1995) (Table 2) were sown in the glasshouse and inoculated with *P. griseola*. Five seeds of each genotype were sown per pot and thinned to three plants. There were three replicates of one pot each (a total of nine plants) for each genotype and the experiment was repeated. The first trifoliate leaf of each plant was inoculated 17 days after planting by spraying 2×10^4 conidia mL^{-1} with a De Vilbiss air compressor at 1/3 horsepower (hp) until plants were wet. Inoculated plants were placed in a humid chamber at approximately 22°C and > 95% relative humidity with a 12 h/12 h dark/light cycle. After 4 days in the humid chamber, plants were put on tables in the glasshouse and the temperature maintained at 24–30°C.

Table 1 Virulence phenotypes of 137 *Phaeoisariopsis griseola* isolates from Central America, Ecuador and Colombia

	Differential genotype[a]												
	Andean						Mesoamerican						
	A	B	C	D	E	F	G	H	I	J	K	L	
Pathotype	1	2	4	8	16	32	1	2	4	8	16	32	Country of origin[b] (number of isolates)
31–15	a[c]	b	c	d	e		g	h	i	j			NIC(1), PRI(1), ELS(2)
31–47	a	b	c	d	e		g	h	i	j		l	CRI(6), HND(1), MEX(2), PAN(2)
31–39	a	b	c	d	e		g	h	i			l	CRI(3), HND(1), PRI(1)
63–47	a	b	c	d	e	f	g	h	i	j		l	CRI(3), HND(2), ELS(1)
63–63	a	b	c	d	e	f	g	h	i	j	k	l	HND(1), NIC(1)
31–43	a	b	c	d	e		g	h		j		l	CRI(2), NIC(2), PAN(1), ELS(1)
23–43	a	b	c		e		g	h		j		l	CRI(1)
31–11	a	b	c	d	e		g	h		j			HND(1), ELS(1)
15–11	a	b	c	d			g	h		j			HND(1)
63–11	a	b	c	d	e	f	g	h		j			HND(1)
63–43	a	b	c	d	e	f	g	h		j		l	HND(2)
15–47	a	b	c	d			g	h	i	j		l	CRI(3), ELS(1)
15–43	a	b	c	d			g	h		j		l	HND(2)
7–43	a	b	c				g	h		j		l	CRI(1), HND(1), NIC(1)

Table 1 *continued*

	Differential genotype[a]												
	Andean						Mesoamerican						
	A	B	C	D	E	F	G	H	I	J	K	L	
Pathotype	1	2	4	8	16	32	1	2	4	8	16	32	Country of origin[b] (number of isolates)
7–47	a	b	c				g	h	i	j		l	CRI(1)
15–55	a	b	c	d			g	h	i		k	l	MEX(1)
15–63	a	b	c	d			g	h	i	j	k	l	HND(1)
15–59	a	b	c	d			g	h		j	k	l	HND(1), MEX(2)
31–59	a	b	c	d	e		g	h		j	k	l	MEX(1)
63–31	a	b	c	d	e	f	g	h	i	j	k		HND(1), NIC(1), ELS(2)
63–27	a	b	c	d	e	f	g	h		j	k		CRI(1), HND(1), ELS(2)
31–27	a	b	c	d	e		g	h		j	k		HND(2), ELS(2)
31–19	a	b	c	d	e		g	h			k		CRI(2), ELS(1)
29–27	a		c	d	e		g	h		j	k		CRI(1)
31–63	a	b	c	d	e		g	h	i	j	k	l	CRI(2), MEX(1), HND(1)
31–55	a	b	c	d	e		g	h	i		k	l	MEX(1)
23–63	a	b	c		e		g	h	i	j	k	l	GTM(1)
31–31	a	b	c	d	e		g	h	i	j	k		CRI(1), MEX(3), ELS(2), NIC(1)
15–31	a	b	c	d			g	h	i	j	k		MEX(1), ELS(1)
31–51	a	b	c	d	e		g	h			k	l	NIC(1)
63–51	a	b	c	d	e	f	g	h			k	l	HND(1), NIC(1)
63–59	a	b	c	d	e	f	g	h		j	k	l	HND(1)
63–19	a	b	c	d	e	f	g	h			k		NIC(1)
63–3	a	b	c	d	e	f	g	h					CRI(1)
31–7	a	b	c	d	e		g	h	i				PRI(2)
63–7	a	b	c	d	e	f	g	h	i				PRI(1)
15–7	a	b	c	d			g	h	i				PRI(2)
5–43	a		c				g	h		j		l	CRI(1)
5–35	a		c				g	h				l	CRI(1)
1–19	a						g	h			k		CRI(1)
1–11	a						g	h		j			CRI(1)
1–3	a						g	h					CRI(1)
7–55	a	b	c				g	h	i		k	l	GTM(1)
7–39	a	b	c				g	h	i			l	CRI(1)
7–19	a	b	c				g	h			k		ELS(1)
7–17	a	b	c				g				k		CRI(1)
5–23	a		c				g	h	i		k		CRI(1)
7–31	a	b	c				g	h	i	j	k		MEX(1)
17–17	a				e		g				k		CRI(1)
17–63	a				e		g	h	i	j	k	l	MEX(1)
12–0			c	d									COL(2)
13–0	a		c	d									ECU(1)
14–0		b	c	d									COL(3), ECU(1)
15–0	a	b	c	d									COL(1)
28–0			c	d	e								COL(1)
29–0	a		c	d	e								COL(1), ECU(1)
30–0		b	c	d	e								COL(2), ECU(1)
31–0	a	b	c	d	e								COL(1), ECU(2)
46–0		b	c	d		f							COL(1)
47–0	a	b	c	d		f							COL(1)
61–0	a		c	d	e	f							COL(1)
62–0		b	c	d	e	f							COL(1)
63–0	a	b	c	d	e	f							COL(4)

[a]Andean differential genotypes: A, Don Timoteo; B, G 11796; C, Bolon Bayo; D, Montcalm; E, Amendoim; F, G5686. Mesoamerican differential genotypes: G, PAN 72; H, G2858; I, Flor de Mayo; J, Mexico 54; K, BAT 332; L, Cornell 49–242.

[b]Country of isolate origin: CRI, Costa Rica; PAN, Panama; GTM, Guatemala; NIC, Nicaragua; PRI, Puerto Rico; ELS, El Salvador; HND, Honduras; MEX, Mexico; COL, Colombia; ECU, Ecuador. The number in parenthesis is the total number of isolates from that country in the designated pathotype.

[c]Lower case letters a to i indicate susceptibility of the respective common-bean differential genotype to the specific pathotype of *P. griseola*.

Table 2 Characteristics of the common-bean differential genotypes used to characterize *Phaeoisariopsis griseola* pathotypes and the binary value used to assign isolates to pathotypes

Differential genotype	Seed size[a]	Bean gene pool	Bean race[b]	Phaseolin type	Binary value assigned to each genotype[c]
A: Don Timoteo	G	Andean	C	H	1
B: G 11796	G	Andean	P	T	2
C: Bolón Bayo	G	Andean	P	H	4
D: Montcalm	G	Andean	NG	B	8
E: Amendoin	G	Andean	NG	T	16
F: G 5686	G	Andean	NG	H	32
G: PAN 72	P	Mesoamerican	M	B	1
H: G 2858	M	Mesoamerican	D	B	2
I: Flor de Mayo	P	Mesoamerican	J	B	4
J: Mexico 54	M	Mesoamerican	J	B	8
K: BAT 332	P	Mesoamerican	M	B	16
L: Cornell 49242	P	Mesoamerican	M	S	32

[a]Seed size: G, large; M, medium; P, small.
[b]Andean common bean races: C, Chile; P, Perú; NG, Nueva Granada. Mesoamerican races: M, Mesoamerica; D, Durango; J, Jalisco.
[c]Binary value used to designate pathotypes of *P. griseola*. For example, if an isolate infects Andean cultivars B and C (binary values 2 and 4) and the Mesoamerican variety K (binary value 16), the race of the isolate is 6–16; the phenotype of this race is bck.

Disease evaluations were conducted 10, 12, 14 and 17 days after inoculation, using a 1–9 visual scale (van Schoonhoven & Pastor-Corrales, 1987) as follows: 1, no symptoms; 3, lesions on 5–10% of the leaf area of the plants; 5, lesions and sporulation on 20% of the leaf area; 7, lesions and sporulation, associated with chlorosis and necrosis, on up to 60% of the leaf area; and 9, lesions, frequently associated with early loss of leaves and plant death, on 90% of leaf area. The disease reaction scores recorded 17 days after infection were used to determine isolate × cultivar compatibility or incompatibility. To designate pathotypes, rating scores of 1–3 were considered incompatible or resistant, while ratings > 3 were considered compatible or susceptible. Pathotype designation was performed by summing the binary values of the differential varieties that were compatible with the respective *P. griseola* isolate (Table 2). For example, for pathotype 31–63 (virulence phenotype abcde-ghijkl), the first value was obtained by summing the binary values of the susceptible Andean differential varieties abcde (1 + 2 + 4 + 8 + 16 = 31) and the second value was obtained by summing the binary values of the susceptible Mesoamerican varieties ghijkl (1 + 2 + 4 + 8 + 16 + 32 = 63) (Pastor-Corrales *et al.*, 1998).

In addition, the area under disease progress curve (AUDPC) was calculated for each inoculated plant from the disease reaction scores at 10, 12, 14 and 17 days after inoculation for each cultivar–isolate combination as:

$$AUDPC = {}_{i}.[(D_i + D_{i-1}) \times (t_i - t_{i-1})]/2$$

where D is the disease score using the 1–9 severity scale and t corresponds to days after inoculation, with i = 10, 12, 14 or 17 days (Shaner & Finney, 1977). AUDPC was used to measure the rate of disease progression, compare aggressiveness of isolates to the various bean genotypes, and ascertain the relative resistance of each plant genotype to the different isolates. Aggressiveness refers to the severity of disease induced by an isolate for a particular host–pathogen interaction (Andrivon, 1993).

DNA extraction

To produce *P. griseola* mycelium, Erlenmeyer flasks (200 mL) containing 60 mL of liquid V8 juice medium were inoculated with 10 disks of 1 cm diameter collected from the edges of actively growing cultures. The cultures were placed on a rotary shaker (115 r.p.m.) and incubated at room temperature (~21°C) for 12 days. Mycelia were harvested by filtration through cheesecloth and freeze-dried. Mycelia (0·25 mg) were ground to a fine powder in liquid nitrogen using a mortar and pestle. Ground mycelium was transferred to a 2-mL microcentrifuge tube and DNA extracted using the SDS method as described by Möller *et al.* (1992). Following washing with 70% ethanol and drying, the pellet was resuspended in 1 × TE buffer containing 10 mg mL^{-1} RNAse A. Tubes were incubated at 37°C for an hour, the DNA re-precipitated with 1/10 volume 3 M NaAc (pH 5·2) and two volumes of 95% ethanol. The pellet was dried and finally resuspended in 0·1 × TE buffer. Electrophoresis through 0·7% agarose gels was used to determine the quality of the extracted DNA. The DNA concentration was measured using a fluorometer (Hoefer® DyNA Quant 2000, Amersham Pharmacia Biotech, Piscataway, NJ, USA) using Lambda DNA as a standard and adjusted to 5 ng μL^{-1} in 0·1 × TE buffer.

RAPD analysis

Seven RAPD primers (OPA2, OPA11, OPA18, OPB15, OPC5, OPF1 and OPH8) (Operon Technologies, Inc.,

Alameda, CA, USA) that produced polymorphisms and yielded reproducible and easily scorable banding patterns with separate DNA extractions and amplifications, were used to amplify DNA from all *P. griseola* isolates. DNA amplification was performed in a MJ Research Thermal Cycler (MJ Research, Watertown, MA, USA) with one cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min, and a final cycle at 72°C for 10 min. Reactions were carried out in 25-μL volumes containing 1 × DNA polymerase buffer (50 mM Tris-HCl [pH 8·5], 2·0 mM $MgCl_2$, 50 mM KCl, 0·1% Triton X–100), 0·2 mM each dNTP, 0·4 μM primer, 1 U AmpliTaq DNA polymerase (PE Biosystems, Foster City, CA, USA) and 20 ng of genomic DNA. DNA amplification products were separated in 1·2% agarose gels at a constant 60 V in 1 × TBE buffer for 5–6 h at room temperature. Fragments were visualized under 300-nm UV light after staining with ethidium bromide, and photographed.

Gel images were captured using the Eagle Eye gel documentation system (Stratagene, La Jolla, CA, USA) and band position was determined using the Quantity One scientific Software, Version 4 (Bio-RAD, Hercules, CA, USA). RAPD fragments of the same size were scored as identical and bands included in the final analysis ranged in size from 0·2 to 2 kb.

Statistical analysis

The disease reaction scores after 17 days were analysed with the Kruskal–Wallis nonparametric variance analysis PROC NPAR1WAY (SAS Institute Inc., Cary, NC, USA) (SAS, 1989) and used to determine compatible and incompatible isolate–cultivar interaction. AUDPCs were calculated for each plant before being averaged over all replicates within a cultivar × isolate pair. The resulting AUDPCs were log-transformed and subjected to analysis of variance (ANOVA) with isolates and cultivars as fixed factors. Duncan's multiple range test was used to compare mean values of the variable log(AUDPC) ($P < 0{\cdot}05$) for each factor: isolates and cultivars and the interaction isolate × cultivar. The epidemic growth rate (r) for each isolate was obtained by linear regression of transformed AUDPC values against time. Duncan's multiple range test was used to compare the regression coefficients and determine the aggressiveness of each isolate.

Multiple correspondence and cluster analysis

Virulence and RAPD data were analysed separately. A data matrix was generated from the virulence phenotypes by considering incompatible interactions (rating 3) as the absence of a virulence marker (0) and compatible interactions (rating > 3) as the presence of a marker (1) and each differential as a different marker. Similarly, RAPD markers were scored as either present (1) or absent (0); only strong and reproducible bands were scored, while faint bands were discarded. Genetic distances between isolates were calculated using Dice's coefficient. The resulting distance matrix was used for multiple correspondence analysis (MCA) using SAS statistics, which determined the positions of isolates on a three-dimensional graph that was drawn by the 'spin' platform of JMP software (SAS, 1995) to provide a visual representation of the associations. The number of clusters based on a consensus among three clustering statistics (local peaks for the cubic clustering criterion and pseudo-F statistics combined with a small value of the pseudo-t^2 statistics and a larger t^2 for the next cluster fusion) were assigned by the average linkage method (SAS). The relative genetic distances between clusters were determined. The cluster consistency index (CCI), which represents percentage matches for each cluster, was obtained with 1000 resampling cycles. A phenogram generated by the unweighted pair grouping by mathematical averaging (UPGMA) (NTSYS-pc version 1·8) using composite data was used to represent the relationship among isolates. Statistical support for phenogram branching in qualitative analyses was obtained using 1000 bootstrapped analysis in WINBOOT (Nelson *et al.*, 1994; Yap & Nelson, 1996). Isolates that were placed in the same cluster in more than 90% of the 1000 resampling and cluster analysis iterations were considered to represent a distinct genetic lineage. Correlation between virulence and RAPD lineages was determined using Spearman's rank correlation coefficients (Snedecor & Cochran, 1967).

Analysis of genotype diversity

Genetic diversity (H), or the average probability that two randomly chosen alleles at a locus are different (Nei, 1973), was calculated for the entire population, as well as for the different groups identified using MCA, employing the following formula:

$$H = 1 - J \text{ and } J = \sum_k x_k^2$$

where x_k is the frequency of the kth allele in the population. A genetic diversity of 1 indicates a very high genetic diversity, where any two alleles at a locus sampled from a population are different, while a genetically uniform population will have a diversity of 0, since any two individuals will be identical.

To estimate gametic phase linkage disequilibrium within *P. griseola* populations, RAPD bands were considered as putative loci with absence or presence of a band as alleles. Linkage disequilibrium was calculated using the GENEPOP version 3·1 computer program (Raymond & Rousset, 1995). The probability test (Fisher's exact test) for each contingency table was performed using the Markov chain method (Raymond & Rousset, 1995). The parameter settings for the Markov chain analysis were 1000 dememorizations, 50 batches and 1000 iterations per batch. An association between loci was considered significantly different from zero if the exact test gave a probability less than 0·05.

Table 3 Number of isolates and pathotypes identified among *Phaeoisariopsis griseola* isolates from each country

Country	Number of isolates	Number of pathotypes	Unique pathotypes[a]
Costa Rica	37	23	13
Guatemala	2	2	2
Honduras	22	17	6
Mexico	14	10	5
Nicaragua	10	9	2
Panama	3	2	0
Puerto Rico	7	5	3
El Salvador	17	12	1
Total	112		32

[a]Unique pathotypes were only recovered in the country of origin.

Results

Distribution of *P. griseola* pathotypes in Central America

Interactions between *P. griseola* isolates and 12 differential bean genotypes were highly significant (Kruskal–Wallis test: $P < 0{\cdot}0001$). Fifty pathotypes were defined among 112 isolates from Central America, while 14 pathotypes were identified among 25 Andean isolates from Ecuador and Colombia (Table 1). None of the pathotypes constituted more than 10% of the sample and most were represented by only one isolate (Table 1). For Central America, 32 (64%) of the pathotypes described were unique to a country, while 18 (38%) were found in two or more countries (Table 3). No pathotype was found in all countries.

Cluster and multiple correspondence analysis separated isolates into three virulence groups (Fig. 1). Group 1 consisted of isolates collected from Andean genotypes in Colombia and Ecuador, which infected only bean differential genotypes of Andean origin. Isolates from Central America were distributed into groups 2 and 3 and these isolates infected some differential genotypes from both the Andean and Mesoamerican gene pools. None of the isolates in group 2 infected either the Andean differential genotype G5686 or the Mesoamerican differential genotype Flor de Mayo. Based on virulence, group 1 (Andean) isolates showed only 42% similarity to Mesoamerican isolates. Within each group, there was no apparent structuring of isolates according to country of origin (Table 1). For example, pathotype 31–39 was recovered from Puerto Rico, Honduras and Costa Rica. Interestingly, two isolates belonging to pathotype 63–63 were recovered from two countries, Honduras and Nicaragua (Table 1). These isolates had overcome the resistance found in all the differential genotypes. Linear regression of transformed AUDPC values against time for each cultivar × isolate interaction to determine epidemic growth rate (r) showed values in the range $r = 0{\cdot}50$–$0{\cdot}99$. Under the experimental conditions described here, isolates from Nicaragua and Honduras were the most aggressive, with pathotype 63–63 having an r-value of 0·99.

Virulence diversity and distribution

With respect to virulence, the reaction of the different isolates varied greatly. Isolates collected from the Mesoamerican region (Middle America and the Caribbean) were the most virulent, infecting both Andean and Mesoamerican differential bean genotypes (Table 1). Isolates collected from the Andean region (Colombia and Ecuador) were the least virulent, inducing susceptible reactions only on

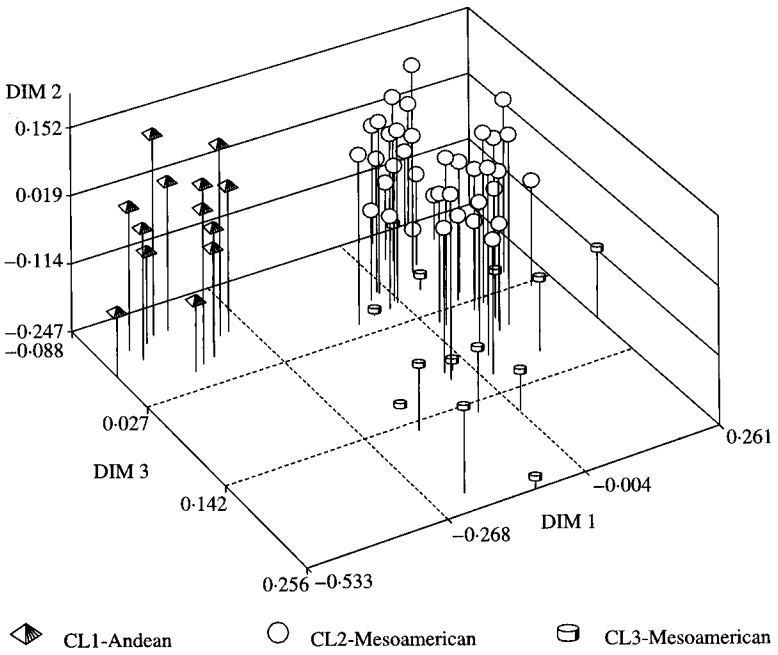

Figure 1 Three-dimensional graph based on multiple correspondence analysis of pathogenicity data from the interaction of *Phaeoisariopsis griseola* isolates on 12 differential bean genotypes and plotted using the spin platform of the JMP program in SAS. Symbols indicate positions of strains within each cluster.

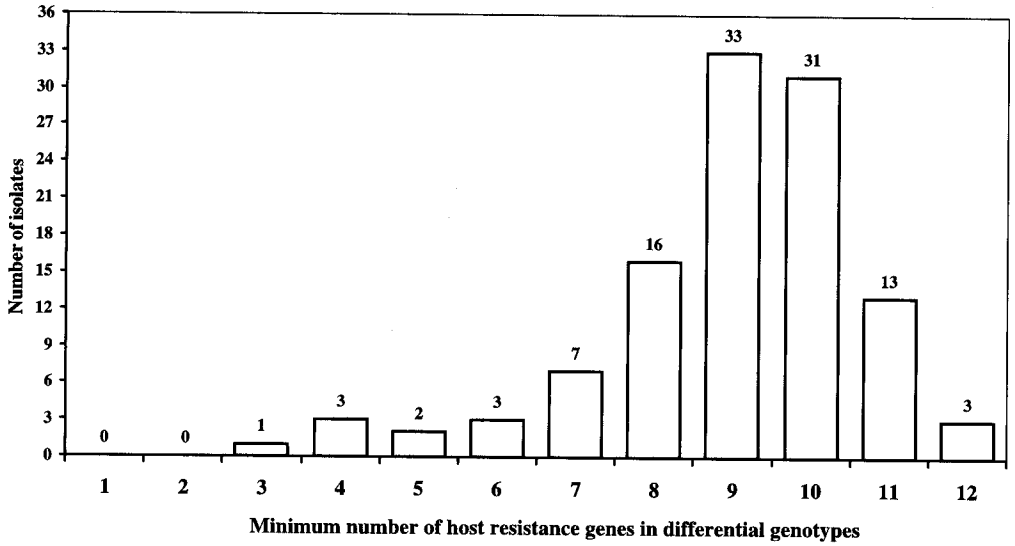

Figure 2 Abundance distribution of virulence genes (avr-) in the overall Central American population of *Phaeoisariopsis griseola*, as determined by compatibility with differential lines.

Andean differential bean genotypes (Table 1). All Mesoamerican *P. griseola* isolates infected PAN 72 (Mesoamerican) and Don Timoteo (Andean), whereas only 25 of the isolates were able to overcome the resistance found in the Andean variety G5686 (Table 1).

No differential variety contained resistance gene(s) effective against all the isolates in this study. Assuming that each differential variety contains a single major gene for resistance, the genetic composition of the *P. griseola* population from Central America is very complex (Fig. 2). The minimum number of virulence genes per pathotype (i.e. the number of host genes the pathotype overcame) varied from 3 to 12. Most isolates (92%) contained complex virulence gene patterns (more than seven virulence genes) and no simple isolates (with zero, one or two virulence genes) were found. Among the common pathotypes, there was no correlation between the number of virulence genes in a pathotype and the frequency of detection. The distribution of the minimum number of virulence genes among all isolates was significantly skewed to the right, with more isolates containing a complex number of virulence genes (Fig. 2).

RAPD analysis

To test the extent of genetic variation in the genome of *P. griseola*, RAPD profiles were generated for the 137 isolates using the selected seven RAPD primers. Examples of the comparative banding profiles of DNA of some isolates using primer H8 are shown in Fig. 3. A total of 54 polymorphic RAPD fragments were detected among *P. griseola* isolates using the seven primers. Cluster and multiple correspondence analyses defined 113 haplotypes among the 137 isolates analysed, distributed into four groups (Fig. 4). Group 1 contained all isolates collected from Andean genotypes from Colombia and Ecuador and the average similarity within this group was 92%. All the Mesoamerican isolates were distributed in groups 2, 3 and 4, with within-group similarities of 84, 65 and 68%, respectively. The Andean isolates (group 1) showed 38% similarity to Mesoamerican isolates (groups 2, 3 and 4), revealing significant differentiation of these two groups according to the two gene pools defined for common bean. A three-dimensional plot of RAPD data (Fig. 4) clearly showed this separation, as well as showing that Mesoamerian isolates were more diverse (average similarity coefficient = 0·64) than Andean isolates (average similarity coefficient = 0·92). Spearman's rank correlation analysis showed a significant correlation (r = 0·60) between *P. griseola* groups (Andean and Mesoamerican) and RAPD markers. However, within each group, there was no correlation (r = 0·021) between profiles generated by RAPD and virulence markers. In addition, isolates belonging to the same pathotype were observed to have completely different RAPD profiles, revealing the lack of congruency between RAPD and virulence phenotypes.

Genetic diversity

Genetic diversity in the *P. griseola* population under study ranged from 0·88 to 0·98 for the RAPD groups defined by MCA analysis. The genetic diversity for the entire population was estimated to be 0·989, showing that the majority (98·9%) of the genotypes present within the *P. griseola* population were unique. Analysis of genetic diversity ascribed 74% of the total diversity to differences between pathotypes in a group, compared with 26% ascribed to differences between them. Similarly, diversity calculated using virulence data ranged from 0·89 to 0·95 for each of the virulence groups defined by MCA analysis and the

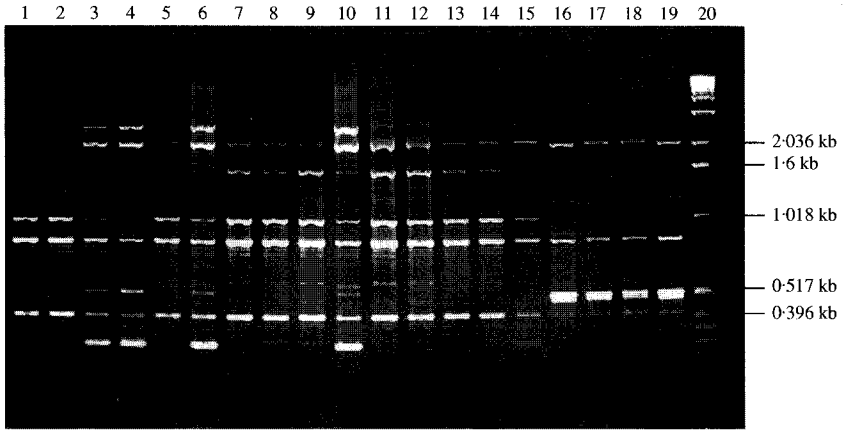

Figure 3 Examples of DNA from some isolates of *Phaeoisariopsis griseola* from Central America, amplified with the random amplified polymorphic DNA (RAPD) primer, H8. Lanes 1–19: *P. griseola* isolates (3 MEX, 4 MEX, 12 MEX, 15 MEX, 16 MEX, 17–1 MEX, 4 NIC, 5 NIC, 6 NIC, 4 HND, 20 HND, 22 HND, 29 HND, 10 CRI, 11 CRI, 5 ELS, 7 ELS, 9 ELS and 17 ELS, respectively); lane 20: 1-kb DNA marker.

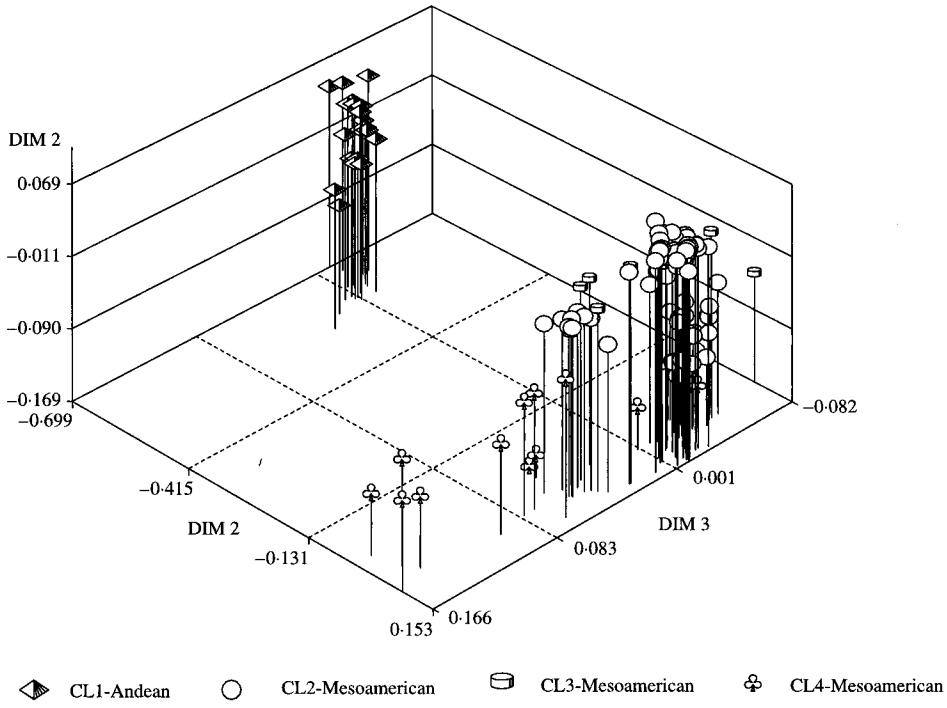

Figure 4 Three-dimensional graph based on multiple correspondence analysis of random amplified polymorphic DNA (RAPD) data for *Phaeoisariopsis griseola* and plotted using the spin platform of the JMP program in SAS. Symbols indicate positions of strains within each cluster.

average pathotype diversity was 0·92. The pathotype diversity for the entire *P. griseola* population was very high (97%). Most of the diversity (81%) resulted from differences within pathotypes in a group, compared with 19% that resulted from differences existing between pathotypes in different groups. Most of the differences between groups resulted from inherent differences between Andean and Mesoamerican isolates.

When considered as a single population, pairwise comparisons of 54 polymorphic RAPD loci gave 1155 (81%) disequilibrium values that were all significantly different from zero (Fisher's exact test, $P < 0{\cdot}05$). The number of contingency tables (loci combinations) was $54(54 - 1)/2 = 1431$. Disequilibrium values could not be calculated for 276 pairs of loci in which one or other of the loci was not polymorphic. When considering the 113 unique genotypes as a single population, no differences were obtained in the number of linked loci. Subdividing isolates or haplotypes into Mesoamerican and Andean groups did not change the number of loci that showed significant linkage disequilibrium (data not shown).

Discussion

In this study, pathotype and RAPD polymorphism were examined to evaluate genetic relatedness and diversity in an attempt to understand the distribution of genetic variation and the virulence structure of *P. griseola* from Central America. Twenty-five isolates collected from bean genotypes belonging to the Andean gene pools and known to belong to the Andean group were included to elucidate the relationship between Andean and Mesoamerican isolates. The results obtained show that isolates from Central America belong to the Mesoamerican group of *P. griseola* and exhibit a wider range of genetic diversity than isolates collected from Andean bean genotypes. MCA of RAPD data grouped 112 Mesoamerican isolates into three putative genetic lineages and Andean isolates into a single group, confirming previous observations that the Mesoamerican group contains a diverse and complex population of *P. griseola* isolates (Maya *et al.*, 1995; Pastor-Corrales & Jara, 1995; Chacón *et al.*, 1997).

Of the 50 pathotypes described among 112 isolates from the Mesoamerican region, not one was common to all the countries under study, despite all countries cultivating beans of Mesoamerican origin. Only 18 pathotypes were recovered from more than one country. Most of the isolates exhibited different virulence patterns, even for isolates originating from the same location, highlighting the existence of a great diversity of pathotypes. An interesting result was the characterization of pathotype 63–63, which overcame resistance in all the differential genotypes. Apart from Honduras and Nicaragua, this pathotype has also been reported in Brazil (Sartorato, 2000). As a gene-for-gene relationship has been demonstrated for the *P. griseola*–common bean pathosystem (Sartorato *et al.*, 1991) the observed virulence diversity reflects significant allelic diversity in virulence genes.

Both virulence and molecular analysis revealed high levels of genetic variation between Central American *P. griseola* isolates. The level of genotype diversity was 99% using RAPD analysis and 97% using virulence markers, showing that the majority of the isolates were unique genotypes. The origin of this wide molecular and virulence diversity is not clear, especially for a pathogen like *P. griseola*, with no known sexual cycle (Liebenberg & Pretorius, 1997). It is known that the basic mechanisms that generate variation in pathogen populations are mutation, recombination (whether sexual or asexual) and migration. This variation is shaped by the forces of selection and genetic drift (Leung *et al.*, 1993; McDonald & McDermott, 1993; Peever & Milgroom, 1994; Zeigler *et al.*, 1995). Major selective forces may be imposed by the degree of specialization in host–pathogen interactions, control measures or more general environmental constraints. These factors act to generate differences in the distribution of phenotypic and genotypic variation among plant pathogen populations that can lead to high genetic variation. It is possible that any one of these factors alone or in combination may be interacting to give rise to new pathotypes, leading to high levels of genetic diversity, as observed in the Central American *P. griseola* population.

Statistical analysis of loci associations found significant gamete phase disequilibrium at all possible pairwise combinations among the 54 RAPD loci in the 137 isolates, showing that *P. griseola* maintains a genetic structure that is consistent with asexual reproduction (McDonald *et al.*, 1995). Gametic phase disequilibrium can arise from founder effects, selection pressures, chromosomal linkage or nonrandom mating, including asexual reproduction (McDonald *et al.*, 1994). The known biology of *P. griseola* indicates that asexual reproduction is a major mode of reproduction for this fungus (Liebenberg & Pretorius, 1997), correlating well with the observed linkage disequilibrium. Because two distinct lineages have been documented for *P. griseola* (Maya *et al.*, 1995; Pastor-Corrales & Jara, 1995; Guzman *et al.*, 1999), recombination might be common within a group, although rare between populations, because of geographical, ecological or biological barriers to gene exchange. Linkage disequilibrium values calculated for each group were significantly different from zero, revealing that *P. griseola* is clonal at all levels. However, the relatively small number of isolates used for the Andean population (25) limits the strength of this conclusion. Nevertheless, these results support the observation that sexual reproduction is absent in *P. griseola*.

Complex virulence patterns were observed among the Central American population of *P. griseola*. These isolates infected both Andean and Mesoamerican bean genotypes and no simple isolates (i.e. isolates with zero, one or two virulence gene combinations) were recovered during this study. Given that the coevolution of *P. griseola* with its common bean host has been shown previously (Maya *et al.*, 1995; Pastor-Corrales & Jara, 1995; Guzman *et al.*, 1999), the results obtained here may suggest the presence of both Middle American and Andean virulence factors within Mesoamerican pathotypes. This hypothesis is supported by the work of Beebe *et al.* (2000), who described a group of bean accessions, considered to be land races from Mexico, that possessed several RAPD bands typical of the Andean beans, and concluded that these were products of introgression from Andean beans. Similar results have been reported for the bean rust pathogen, *Uromyces appendiculatus* (Sandlin *et al.*, 1999) and the bean anthracnose pathogen, *Colletotrichum lindemuthianum* (Kelly, 1995; Balardin & Kelly, 1998), where pathotypes with virulence factors to both host gene pools were described. Because of extensive common-bean breeding activities, some varieties have some level of introgression in them and the pathogen has evolved to colonize and adapt to such varieties.

No correlation was observed between pathogenicity patterns on bean differential genotypes and RAPD data. *Phaeoisariopsis griseola* isolates belonging to the same pathotype (i.e. with similar virulence phenotypes) were observed to have very different molecular profiles. These results are consistent with other findings that isolates of the same pathotype are not necessarily closely related (Jacobson & Gordon, 1990; Woo *et al.*, 1996; Sicard

et al., 1997; Balardin *et al.*, 1999). The results of the present study show that virulence must have arisen independently in different geographical areas at different times and independent of the genetic background.

The vast virulence diversity of the ALS pathogen in Central America means that using a single location to test the resistance of a developed variety or source of ALS resistance is not sufficient, since different pathotypes were described in different locations and countries. Pyramiding resistance genes into appropriate backgrounds is a strategy with potential for maintaining long-term durable disease resistance (Nelson, 1978; Leung *et al.*, 1993; Kelly, 1995; Young & Kelly, 1996). For example, the bean anthracnose differential genotype G2333 contains three resistance genes (Co-4^2, Co-5 and Co-7) and, so far, only one pathotype from Costa Rica (CIAT, 1995) has been found to infect this genotype, demonstrating the effectiveness of pyramiding resistance genes (Pastor-Corrales *et al.*, 1994; Young *et al.*, 1998).

The information gained in this study has significant implications for regional ALS resistance breeding and resistance gene deployment. The pathogen structure of *P. griseola* appears consistent with asexual reproduction, and resistance-gene pyramiding into well-adapted bean varieties constitutes an appropriate deployment strategy that is most likely to give durable resistance. In addition, the information on pathogen population structure and distribution of pathotypes obtained for Central America will help in identifying sources of resistance and targeting or deploying resistance genes.

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