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Isozyme variability among isolates of *Phaeoisariopsis griseola* in southern Africa

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Angular leaf spot caused by *Phaeoisariopsis griseola* is an economically important disease of beans (*Phaseolus vulgaris*) in southern Africa. The success of local programmes breeding for resistance to this disease depends to a large extent on the genetic variation within the pathogen population. To assess variability within the pathogen, 28 isolates of *P. griseola* from various localities were compared using isozyme analysis by means of starch gel electrophoresis. Thirteen loci were identified in 10 enzyme systems. Using UPGMA, three electrophoretic types were detected. The most common type included the South African isolates, namely seven from the Mpumalanga and KwaZulu-Natal provinces, respectively, 10 from Malawi, and one from Portugal. Two isolates from Bembeke in Malawi, and one from the Netherlands, differed from the rest. An isolate of *Phaeoramularia angolensis*, used as an outgroup, differed from the *P. griseola* isolates in all enzyme systems tested. The high homology of banding patterns among isolates of *P. griseola* from southern Africa suggests the local population to be uniform.

INTRODUCTION

Angular leaf spot, caused by the asexual state of the fungus *Phaeoisariopsis griseola* (Sacc.) Ferraris (teleomorph unknown), is a major disease of beans (*Phaseolus vulgaris*) in tropical and subtropical areas (Thurston, 1989; Deighton, 1990; Saettler, 1991). *Phaeoisariopsis griseola* infects numerous crops, including common bean, lima bean, scarlet runner bean, tepary bean, black gram, pea, and cowpea (Saettler, 1991). The disease affects foliage and pods of beans throughout the growing season and is particularly destructive in areas where warm, moist conditions are accompanied by abundant inoculum from infested plant residues and contaminated seed (Saettler, 1991). Field symptoms are generally observed soon after flowering, or as plants approach maturity. Leaf lesions are the most conspicuous, and start as small, brown or grey spots that develop into angular, necrotic lesions delimited by vascular strands. Lesions eventually enlarge, coalesce and cause defoliation of plants. Most crop losses result from premature defoliation (Correa-Victoria *et al.*, 1989; Saettler, 1991). Circular to elliptical red-brown lesions can develop on pods, whereas browning of stems has also been attributed to this disease.

In southern Africa, *P. griseola* is a limiting factor of bean production in the more humid areas. In these regions situated towards the subtropical areas in the east, annual losses in yield and quality have been estimated between 20 and 40%, with evidence of losses up to 80% in certain years (A.J. Liebenberg, Grain Crops Institute, South Africa, personal communication, 1995). Control measures include seed treatment, crop rotation, removal of infected crop debris, foliar fungicides, and the breeding of resistant cultivars (Correa-Victoria, 1987; Saettler, 1991).

Isolates of the pathogen vary considerably regarding their virulence spectrum, and 14 pathotypes have been identified worldwide (Correa-Victoria, 1987). Using isozyme analysis, 55 isolates of *P. griseola* from Latin America, the United States and Africa were divided into two groups (Correa-Victoria, 1987). All African isolates displayed the same electrophoretic pattern and were associated with large-seeded bean cultivars originating in the Andean region of

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South America. A second electrophoretic pattern was associated with small-seeded bean cultivars from Central and North America. In a recent study using random amplified polymorphic DNA (RAPD) markers, 62 isolates of *P. griseola* from Malawi, the United States and Brazil were similarly divided into two major groups corresponding with large and small-seeded cultivars, respectively (Guzmán *et al.*, 1995).

For local breeding programmes to succeed, the extent of genetic diversity within the pathogen population must be determined. Electrophoretic patterns of soluble enzymes represent a direct manifestation of the genome of an organism and can be of considerable value to determine genetic variation among a number of enzyme loci (Micales *et al.*, 1992). The objective of this study was to screen isozyme activity in isolates of *P. griseola* as a measure of genetic variation within the pathogen population.

MATERIALS AND METHODS

Isolates used and sample preparation

Isolates of *P. griseola* used in this study are listed in Table 1. *Phaeoramularia angolensis* (de Carvalho & O. Mendes) P.M. Kirk, another cercosporoid fungus with densely fasciculate conidiophores (Kirk, 1986a), isolated from citrus leaves (CPC 751), was included as outgroup. Single-spored isolates were grown on potato-dextrose agar (PDA) (Difco) on which cellophane had been placed to facilitate harvesting of the mycelium for electrophoresis. The harvested mycelium was ground with a mortar and pestle in liquid nitrogen, and transferred to 2-mL Eppendorf tubes containing 0·5 mL extraction buffer (1·21 g Trizma Base, 0·292 g EDTA, 38 mg NADP, and 10 mg PVP/L distilled H_2O) (Petrunak & Christ, 1992). All fungal isolates used in the present study are maintained in the culture collection of the Department of Plant Pathology at the University of the Orange Free State.

Starch gel electrophoresis

The mycelium–buffer mixture was centrifuged at 4000 r.p.m. for 2 min. The resulting supernatant was absorbed onto thick filter paper wicks (4 × 10 mm). Loaded wicks were inserted into a sample slot, 2 cm from the cathodal end of the starch gel (12% w/v). After completion of electrophoresis, each gel was cut horizontally

Table 1 Electrophoretic types (ETs) found in 28 isolates of *Phaeoisariopsis griseola* and one isolate of *Phaeoramularia angolensis* according to 10 enzyme systems[a]

Origin	Isolate number	ET
South Africa		
Greytown	RSAPg92GT9, RSAPg93GT16	1
Cedara	RSAPg92CE20, RSAPg93CE1, RSAPg93CE13, RSAPg94CE4	1
Vulindlela	RSAPg93VU22	1
Delmas	RSAPg91DE12	1
Ohrigstad	RSAPg91OS11, RSAPg91OS23	1
Lydenburg	RSAPg91LY33, RSAPg93LY28, RSAPg93LY29	1
Komatipoort	RSAPg93KP32	1
Malawi		
Bunda	MPg92BM10, MPg92BM16,MPg93BM31	1
Dedza	MPg92DM14, MPg93DM42, MPg94DM1	1
Champira	MPg91CM4, MPg92CM13	1
Chitedze	MPg94HM2	1
Bembeke	MPg93KM43, MPg93KM44 MPg94KM5	2
Portugal	CBS 194.47	1
The Netherlands	CBS 880.72	3
Zimbabwe	CPC 751	4[b]

[a] Enzyme systems used were PGI, LAP, PEP(GL), PEP(LGG), AK, MDH, ACP, LDH, CAT, EST.

[b] *Phaeoramularia angolensis* isolated from citrus.

into five slices and stained with specific enzyme stains (O'Malley *et al.*, 1980; Micales *et al.*, 1986; Selander *et al.*, 1986). Gels were assayed for acid phosphatase (ACP), adenylate kinase (AK), aconitase (ACO), alcohol dehydrogenase (ADH), catalase (CAT), esterase (EST), fluorescent esterase (FLE), glucose-6-phosphate dehydrogenase (G6P), glucose-phosphate isomerase (GPI), β-glucosidase (β-GLU), glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), peptidase (PEP; using leucyl-glycyl-glycine [LGG], leucyl-tyrosine [LT], and glycyl-leucine [GL] as substrates), peroxidase (PRX), phosphoglucose isomerase (PGI) and sorbitol dehydrogenase (SDH). All electrophoretic patterns were confirmed at least once depending on the resolution of the bands.

Data analysis

Allozymes were designated by their mobilities relative to the most common allozyme, which was designated as 100. From the resultant genotypic information, allele frequencies and Nei's unbiased genetic distances (Nei, 1978) were calculated and the resultant matrix of distances were analysed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to produce a dendrogram (Rohlf, 1993).

RESULTS

Enzyme activity was detected in mycelium extracts of *P. griseola* for 13 enzyme-staining procedures analysed. Ten of these enzymes (Table 2) provided consistent results with adequate isozyme resolution for accurate assessment. Enzyme-staining protocols that resulted in a less than desirable degree of staining intensity or uniformity were: ACO, ADH, FLE, G6P, GPI, β-GLU, GDH, PEP(LT), PRX, and SDH. The gene products of 13 loci were detected in the 10 enzyme systems. All loci were polymorphic, except for ACP which was monomorphic. Three electrophoretic types were detected for the *P. griseola* isolates. Electrophoretic type 1 was the most common, and included all South African isolates, 10 Malawian isolates and one isolate from Portugal (CBS 194.47). Two isolates of *P. griseola* from Malawi (MPg93KM43, MPg93KM44) and one from the Netherlands (CBS 880.72) differed from the rest, while *Phaeoramularia angolensis* differed from all isolates of *P. griseola* in all enzyme systems tested (Fig. 1). The isozyme banding patterns for LAP, CAT, and EST are shown in Table 3. A UPGMA phenogram, illustrating genetic distance and relationships among electrophoretic types of the 28 *P. griseola* isolates and the *Phaeoramularia* outgroup, is presented in Fig. 2.

DISCUSSION

Due to the common occurrence of angular leaf spot on beans in southern Africa, breeding for resistance against this disease is important. However, little progress can be anticipated if the extent of genetic variation within local populations of the fungus is not known. The

Table 2 List of enzymes and buffer systems which gave clear resolution of bands

Enzyme	Abbreviation	E.C.	Buffer system
Phosphoglucose isomerase	PGI	5.3.1.9	RW[a]
Leucine aminopeptidase	LAP	3.4.11.1	RW
Peptidase (glycyl-leucine)	PEP(Gl)	3.4.x.x	TC[b]
Peptidase (leucyl-glycyl-glycine)	PEP(LGG)	3.4.x.x.	TC
Adenylate kinase	AK	2.7.4.3	AC[c]
Malate dehydrogenase	MDH	1.1.1.37	AC
Acid phosphatase	ACP	3.1.3.2	RW
Lactate dehydrogenase	LDH	1.1.1.27	AC
Catalase	CAT	1.11.1.6	RW
Esterase	EST	3.1.1.1	RW

[a] Discontinuous buffer according to Ridgway *et al.* (1970), pH 8·5, 75 A, 4 h.
[b] Discontinuous buffer according to Poulik (1957), pH 8·7, 75 A, 4 h.
[c] Continuous buffer according to Clayton & Tretiak (1972), pH 6·1, 90 A, 3 h.

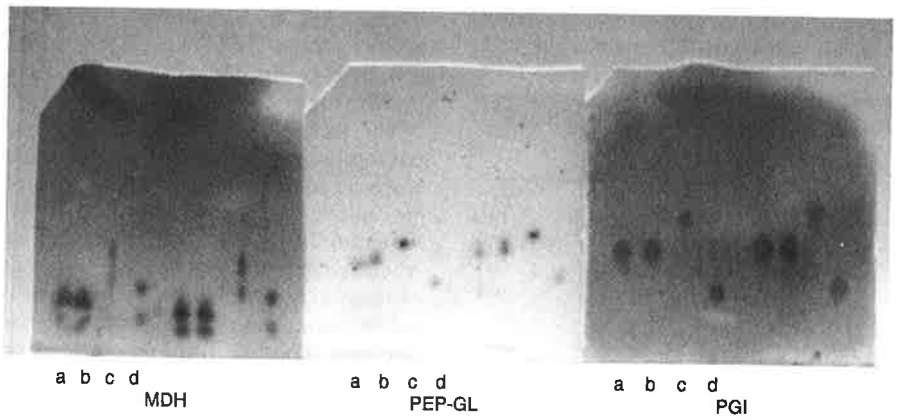

Fig. 1 Three enzyme systems (MDH, PEP-GL and PGI), each showing from left to right, lanes of a representative South African (a) and two Malawian (b,c) isolates of *Phaeoisariopsis griseola,* and *Phaeoramularia angolensis* (d). On each gel the lanes not numbered represent a replicated set of isolates a to d.

availability of pathotypes, representative of the complete pathogenicity spectrum in southern Africa, should greatly enhance the effectivity of germplasm screening and selection for resistance. The use of isozymes as markers for estimating the amount of diversity within a fungal population is well documented (Micales *et al.*, 1986; McDonald & McDermott, 1993). Using this technique, several workers have presented evidence for the delineation of taxa at a

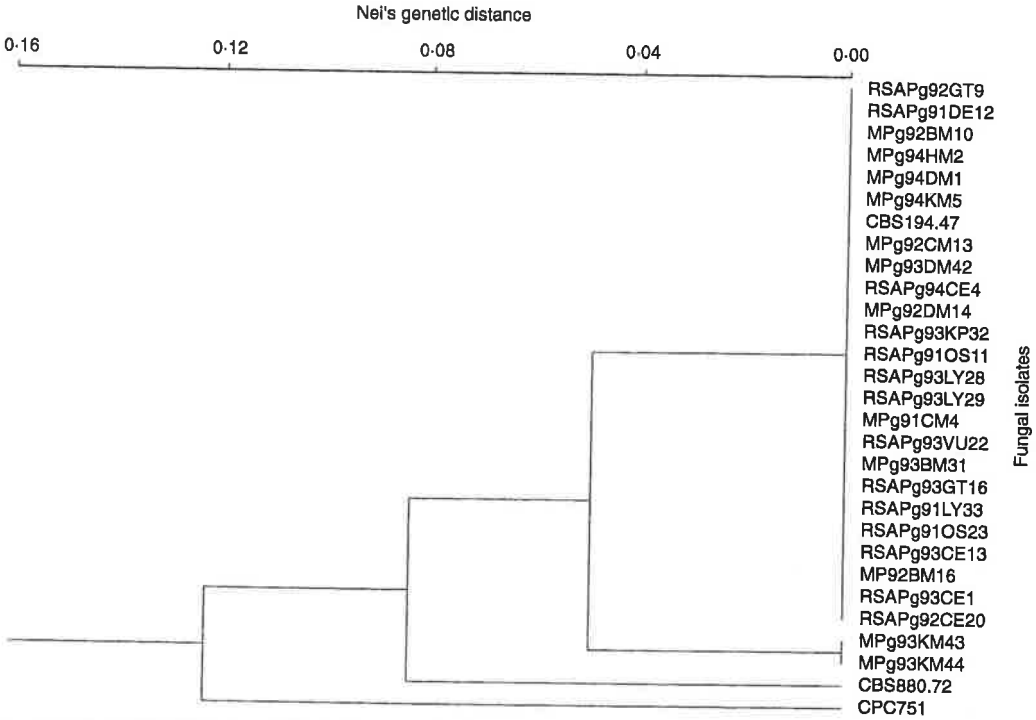

Fig. 2 A UPGMA phenogram illustrating genetic distances and relationships among electrophoretic types of 28 *Phaeoisariopsis griseola* isolates and one isolate of *Phaeoramularia angolensis.*

Table 3 Electrophoretic phenotypes of leucine aminopeptidase (LAP), catalase (CAT) and esterase (EST) identified in 28 *Phaeoisariopsis griseola* isolates (EP1, EP2, EP3) and one isolate of *Phaeoramularia angolensis* from citrus (EP4) by means of starch gel electrophoresis.

Locus	Allele	EP1	EP2	EP3	EP4
LAP	105		—		nd
	100	—		—	nd
CAT	100	—	—		—
	88			—	
	69	—			
	31			—	
EST1	100	—	—		—
	91	—	—		
	76			—	—
EST2	110		—	—	
	100	—	—		—
	95			—	
EST3	106		—	n	
	100	—		n	—
	94		—	n	—

Allele values indicate relative electrophoretic mobility, with 100 designating the most common allele; n, null allele; nd, not detected.

subspecies level. Bosland & Williams (1986) distinguished pathotypes of *Fusarium oxysporum* Schltdl. and Burdon & Marshall (1981) detected variation among different formae speciales of species of *Puccinia* Pers. Furthermore, Burdon & Roelfs (1985) discerned several virulence phenotypes of *P. graminis* Pers.:Pers. f. sp. *tritici* Eriks. & Henn. according to isozyme electrophoretic patterns. Bielenin *et al.* (1988) also distinguished subgroups of *Phytophthora megasperma* var. *medicaginis* Kuan & Erwin, that differed in pathogenicity, using isozyme analysis. In *P. griseola*, however, Correa-Victoria (1987) found no relationship between 14 pathotypes and electrophoretic patterns.

The electrophoretic patterns of soluble enzyme extracts obtained from all South African and 10 Malawian isolates of *P. griseola* showed no variation in the number and position of bands. Furthermore, the presence of two Malawian isolates, clearly different from all other southern African isolates, is consistent with findings that two groups of *P. griseola* co-exist with their respective host cultivars in Malawi (Guzmán *et al.*, 1995). In accordance with these results our data suggest that isolates from one group only, associated with the most commonly cultivated large-seeded bean cultivars, were detected in South Africa. The identity of isolate CBS 880.72, that did not correspond with either of these two groups, was not verified.

The lack of genetic variation within fungal pathogens has previously been correlated with various factors. Newton (1987) reported that a specialized, obligate parasite should exhibit little variation due to the uniformity of its substrate, whereas facultative pathogens, which are more exposed to diverse substrates and environments, will be variable. In South Africa, *P. griseola* has not been reported from, or observed on, hosts other than *P. vulgaris*. However, several *Desmodium*, *Phaseolus* and *Vigna* spp., as well as *Dolichos lablab*, *Glycine max*, *Hibiscus esculentus* and *Pisum sativum*, have been listed as host plants for *P. griseola* (Kirk, 1986b). The degree of sexual reproduction within a population or species is also directly related to the level of genetic variation in that population or species (Kendrick, 1992). In this regard, the lack of variation in *P. griseola* could partly be attributed to the absence of the teleomorph. Old *et al.* (1984) ascribed a lack in polymorphism to the recent introduction of a pathogen in an area. In South Africa, however, the first specimens of this fungus were collected by J.W. Watts from Barberton in the Mpumalanga province in 1906, and by J.B. Pole-Evans in KwaZulu-Natal in 1912. Voucher specimens (PREM 15, PREM 2387 and PREM 11387) of these collections were lodged at the National Collection of Fungi in Pretoria.

Given the uniform electrophoretic pattern exhibited by the South African population, it seems doubtful that isozyme analyses can be applied to detect specific virulence characteristics in local *P. griseola* isolates. Although the isolates used in the present study were selected to represent geographically different areas, it is also possible that the sample was too small to detect the presence of the second group among South African isolates. In view of this, a larger population of local and foreign isolates should be screened to define the true extent of genetic variation within *P. griseola*. More sensitive DNA techniques should therefore be evaluated as a possible means for describing pathogenic variation in the southern African population of *P. griseola*.

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