Preliminary Study of Virulence and Isozymic Variation in Natural Populations of *Colletotrichum gloeosporioides* from *Stylosanthes guianensis*

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ABSTRACT

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Variation for nine virulence and four electrophoretic characters was determined for 69 samples of *Colletotrichum gloeosporioides* collected in five natural stands of its host, *Stylosanthes guianensis*, in South America. Virulence phenotypes varied from four in the Quilichao population to 22 in the Darien population. Isozymic diversity, on the other hand, was limited to a few phenotypes. In contrast to some studies of other fungal pathogens, tight associations between isozyme phenotypes and virulence groupings were not evident. However, the limited number of isozyme phenotypes present and the linkage disequilibrium detected between alleles at several enzyme loci indicate a high level of inbreeding or clonal reproduction in these populations of *C. gloeosporioides*.

Stylosanthes guianensis (Abl.) Sw., common stylo, is a morphologically diverse tropical to subtropical pasture legume species naturally distributed from latitude 23°N to 27°S throughout Central and South America (14,21). As one of the most promising *Stylosanthes* species, it has been a source of several forage cultivars in Australia (9,21) and South America (19).

Anthracnose, caused by the fungus *Colletotrichum gloeosporioides* (Penz.) Sacc. is the most widespread and damaging disease of *S. guianensis* worldwide (10). Dry matter losses from 65 to 100% have been recorded in *S. guianensis* in Colombia (10). Comparisons of virulence of many isolates of *C. gloeosporioides* from *S. guianensis* collected principally in tropical South America and Australia have shown considerable variation (J. M. Lenné and J. A. G. Irwin, *unpublished data*). Most of the variation in virulence has been found in isolates collected in Brazil, Colombia, and Peru (J. M. Lenné, *unpublished data*). Both quantitative and qualitative differences in virulence among isolates of *C. gloeosporioides* sampled from a natural population of *S. guianensis* in Colombia were detected (13). One isolate type was uniformly virulent across the population of *S. guianensis*, whereas a second type showed large differences in virulence to members of the population.

Studies of the genetic structure of plant pathogen populations always have been hampered by difficulties in extrapolating beyond observed differences in pathogenicity scored at a phenotypic level to the underlying genetic basis for these differences. This problem is particularly acute in situations involving poorly understood host-pathogen systems. In these associations, concurrent surveys of electrophoretic variation may well provide useful information upon which observed patterns of virulence may be interpreted. In this respect, electrophoretic analysis of isozymes has provided valuable information on the variability and population biology of a range of fungal pathogens (4) including *Pyricularia oryzae* Cavara (12), various *Puccinia* species (2,3,5,6), *Phytophthora cinnamoni* Rands (18), and *Rhynchosporium secalis* (Oudem.) J. J. Davis (15).

The objective of the present study was to combine the results of a survey of the virulence of 69 isolates of *C. gloeosporioides* collected from five natural populations of *S. guianensis* growing in Colombia and Peru with an assessment of soluble enzyme variation of the same pathogen samples.

MATERIALS AND METHODS

Collection sites. *S. guianensis* was the dominant legume at all five sites examined; however, its presence varied from rare to abundant (Table 1). The incidence of anthracnose varied from low (1–10% of plants infected) at Calzada to high (greater than 40% infected) at Darien, Quilichao, and Tarapoto (Table 1). However, anthracnose severity according to a 1–5 rating scale was low (1) to moderate (2–3) except at Darien where some severely diseased plants (>4) were observed (Table 1). The anthracnose rating scale is discussed in detail in the section about virulence testing.

Collection, isolation, and maintenance of isolates of *C. gloeosporioides*. A total of 69 isolates of *C. gloeosporioides* was collected from the five natural populations of *S. guianensis*. Sample numbers per population varied from nine (Tarapoto) to 28 (Darien) (Table 2). At all sites, isolates were collected during the mid to late wet season, the period of greatest anthracnose incidence and severity. Each site was observed for at least two seasons before isolate collection. At each site, stem lengths of 1–2 cm with well-developed anthracnose lesions were collected from single plants of *S. guinanenis* and placed in paper bags. In the laboratory, small pieces of lesioned tissue were surface sterilized in 1% NaOCl for 10 min, rinsed several times in sterile distilled water, and plated onto oatmeal agar (OMA) according to the method of Lenné and Sonoda (11). Developing colonies were purified, to ensure that each was a single genotype, and then stored in vials of sterile distilled water at 20 C (1). Under these conditions, isolates were stable and retained their pathogenicity.

Virulence testing of isolates of *C. gloeosporioides*. Inoculum for the virulence testing procedure was produced by culturing isolates of *C. gloeosporioides* on OMA for 12 days at 25 C with a 12-hr photoperiod (11). Conidia were washed from the cultures, filtered through cheesecloth, and suspended in sterile distilled water (10^6 conidia ml^{-1}) before being sprayed onto 35-day-old seedlings of nine lines of the differential set of *S. guianensis* (Centro Internacional de Agricultura Tropical [CIAT] accession numbers 13, 15, 17, 136, 184, 1283, 1927, 1949, and 1951 [7]). Inoculated seedlings were enclosed in moist plastic bags and incubated at 22–28 C under a 12-hr photoperiod for 48 hr. Disease severity ratings were made 10 days after inoculation according to a 1–5 scale where: 1 = no disease; 2 = few (1–10), small (1–2 mm

TABLE 1. Sites of collection of isolates of *Colletotrichum gloeosporioides* from natural populations of *Stylosanthes guianensis*

Site	Country	Longitude	Latitude	(m)	Abundance of *S. guianensis*[a]	Incidence of anthracnose[b]
Darien	Colombia	76°25′W	3°55′N	1,729	Abundant	High
Quilichao	Colombia	76°31′W	3°06′N	990	Rare	High
Calzada	Peru	77°02′W	6°00′S	900	Mod. abundant	Low
Lamas	Peru	76°31′W	6°28′S	770	Abundant	Moderate
Tarapoto	Peru	76°19′W	6°32′S	460	Mod. abundant	High

[a]Rare = <1 plant per 50 m^2; moderately abundant = 1–10 plants per 50 m^2; abundant = >1 plant per 1 m^2.
[b]Low = 1–10% of plants with anthracnose; moderate = 11–40% of plants with anthracnose; high = >40% of plants with anthracnose.

TABLE 2. Isozyme and virulence variation detected in five populations of *Colletotrichum gloeosporioides* collected from five separate natural populations of *Stylosanthes guianensis*

Population	No. of isolates	No. of phenotypes		No. of sexual isolates
		Virulence	Isozyme	
Darien	28	22	6	2
Quilichao	10	4	1	2
Tarapoto	9	9	1	2
Calzada	11	7	1	0
Lamas	11	10	1	3

in diameter) lesions on leaves, less than 25% of stem with lesions; 3 = moderately abundant (11–20), small to large (greater than 4 mm in diameter) lesions, 25–50% of stem with lesions, less than 10% defoliated; 4 = abundant (greater than 20), mostly large lesions, 50–75% of stem with lesions and dieback, 10–40% defoliated; 5 = plant death (11). Isolates were classified into distinct phenotypes on the basis of their reaction to the nine differential lines of *S. guianiensis*: resistant—rating = 1; intermediate—rating = 2; and susceptible—rating = 3 or greater. Virulence tests of all isolates were repeated at least twice to verify results.

Preparation of isolates for electrophoresis. The reproductive behavior of the isolates of *C. gloeosporioides* used in this study was variable. Isolates variously produced asexual conidia only (anamorphic) or combined sparse conidial production with the production of either a profusion of sexual spores (basically teleomorphic), vegetative mycelium (basically anamorphic), or a combination of both. However, when grown on OMA, the majority of isolates (55 in 69) produced abundant asexual conidia in acervuli on the surface of the colony. The conidia were harvested from these structures by carefully scraping the surface of colonies with a spatula. Conidia were washed once with buffer solution (50 mM phosphate, pH 7.0, containing 1.5 mg ml^{-1} dithiothreitol), then placed in a few drops of this buffer and frozen in liquid nitrogen. For nine teleomorphic isolates, sexual spores were collected in a similar manner. For five vegetative isolates, mycelium was grown at room temperature (20–22 C) in liquid shake culture of modified V-8 prepared by mixing 354 ml of V-8 juice with 5 g of $CaCO_3$, centrifuging this solution at 4,000 rpm for 20 min, decanting and diluting the supernatant 1:4 with deionized distilled water, and later sterilizing by autoclaving. After 10 days, the resultant mycelium was washed with distilled water, blotted dry between sheets of paper toweling, and then frozen in liquid nitrogen.

In five cases, enzyme extracts were obtained from both mycelium and spores of the same pathogen culture. These subsequently were subjected to electrophoresis to determine the consistency of expression and electrophoretic mobility of enzymes extracted from different tissue types. In no case were any mobility differences observed between samples from the same pathogen isolate.

Electrophoretic analysis of isozymes. Horizontal starch-gel electrophoresis was performed on homogenized extracts of this material using one of two buffer systems (system A: electrode buffer—0.4 M sodium citrate pH 8.0, and gel buffer—5.0 mM, histidine, pH 8.0; system B: electrode buffer—0.3 M borate, 0.1 M sodium hydroxide, and gel buffer—3.0 mM citrate, 15.2 mM Tris). For system A, electrophoresis was conducted for 5 hr, and for system B it was allowed to proceed until the borate front had migrated 9 cm from the sample slot. After electrophoresis, each gel was cut horizontally into three slices, and the anodal portion of the gel was assayed for 12 different enzyme systems. Four enzymes gave clearly resolved bands that could be scored reliably: phosphoglucose isomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), superoxide dismutase (SOD, EC 1.15.1.1), and triosephosphate isomerase (TPI, EC 5.3.1.1)—all assayed on system A. Resolution of bands of activity was poor or unreliable in the remaining eight enzyme systems (acid phosphatase, aconitate hydratase, dihydrolipoamide reductase, fumarase, hexokinase, menadione reductase, peptidase, and umbelliferyl esterase). Enzyme assays were as cited in Collins et al (8). Each pathogen isolate was tested at least twice for the four reliable enzymes.

RESULTS

The number of virulence phenotypes per population based on the response of nine differential lines of *S. guianensis* varied from four in the Quilichao population to 22 in the Darien population (Table 2). Results obtained for the 28 isolates collected at Darien (Table 3) are representative of the range of virulence phenotypes encountered.

All 69 isolates of *C. gloeosporioides* collected also were surveyed for electrophoretic variation in four enzyme systems. For each system, a single band of activity, tentatively attributed to a single locus, was scored. No isolate exhibited evidence of more than a single allele at any of the loci scored. No differences were detected within an isolate in the electrophoretic mobility of enzyme bands derived from conidia, sexually produced spores, or mycelium. Similarly, no differences were detected in the enzymatic activity of conidia derived from different aged (13- and 26-day-old) cultures. Multiple alleles at a single locus were designated "a," "b," "c" in order of decreasing electrophoretic mobility.

Three alleles were detected at the PGI, PGM, and SOD loci and two at the TPI locus. However, these were not distributed at random among the isolates and only seven multilocus isozyme phenotypes were identified (Table 4). The four populations represented by small sample sizes each possessed a single phenotype. The phenotype for Calzada was unique (T4: PGI b, PGM b, TPI b, SOD b), whereas the Lamas, Quilichao, and Tarapoto populations shared a common multilocus pattern (T5: PGI b, PGM c, TPI b, SOD a).

In contrast, six distinct multilocus isozyme phenotypes were detected in the Darien population (Table 4). Three of these (T5, T6, and T7) were present at frequencies exceeding 10%. The remaining phenotypes (T1, T2, and T3) were represented by single isolates only. The distribution of these multilocus isozyme phenotypes with respect to the virulence phenotypes of the *C. gloeosporioides* collected at Darien is given in Table 3. In several cases, isolates possessing the same virulence phenotype had different multilocus isozyme patterns (for example, virulence groupings 5, 8, and 9). Conversely, the one multilocus isozyme phenotype (T6) was associated with 13 different virulence patterns.

The results of X^2 tests for association between alleles at the PGI, PGM, and SOD loci in the Darien population are given

TABLE 3. Virulence and isozyme multilocus phenotypes for 28 isolates of *Colletotrichum gloeosporioides* collected from a natural population of *Stylosanthes guianensis* growing at Darien, Colombia

	Virulence phenotypes[b]																					
Differential[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
13	A	A	A	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	A	V	V	V
15	I	A	I	V	V	V	V	V	V	V	V	A	A	A	V	A	A	A	A	A	A	A
17	I	A	I	V	V	V	V	V	V	V	V	V	V	V	V	V	V	A	A	A	A	A
136	I	I	A	A	A	V	V	V	V	V	V	V	V	V	V	V	V	A	V	A	V	A
184	I	I	I	I	A	V	V	V	V	V	V	V	V	A	A	V	V	A	A	A	A	I
1283	I	I	I	I	I	A	V	A	I	A	I	I	A	I	A	I	A	I	A	A	I	I
1927																						
1949	I	I	I	I	I	A	V	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
1951	A	A	I	A	A	A	V	V	V	A	A	V	V	V	V	A	A	V	V	V	A	A
No. of isolates	1	2	1	1	2	1	1	2	2	3	1	1	1	1	1	1	1	1	1	1	1	1
Isozyme phenotype	T6	T6	T5	T6	T5 T6	T6	T6	T6 T7	T5 T7 T2[c]	T5 T5	T6	T6	T1[c]	T6	T6	T6	T5	T6	T3	T7	T5	T7

[a] Centro Internacional de Agricultura Tropical accession numbers for the differential set of *S. guianensis*.
[b] A, avirulence; V, virulence; I, intermediate reactions.
[c] Sexual isolate.

TABLE 4. Allelic identity of the multilocus isozyme phenotypes and their frequency in the five sampled populations of *Colletotrichum gloeosporioides*

Multilocus phenotype	Enzyme locus and allele				Frequency in population[y]				
	PGI	PGM	TPI	SOD	Cal.	Dar.	Lam.	Qui.	Tar.
T1	a[z]	a	a	b	...	0.04	...	...	...
T2	a	a	b	b	...	0.04	...	...	...
T3	b	b	b	a	...	0.04	...	...	...
T4	b	b	b	c	1.00	...	...	...	...
T5	b	c	b	a	...	0.25	1.00	1.00	1.00
T6	b	c	b	c	...	0.50	...	...	...
T7	c	b	b	a	...	0.14	...	...	...

[y] Cal., Calzada; Dar., Darien; Lam., Lamas; Qui., Quilichao; Tar., Tarapoto.
[z] Alleles are designated in order of decreasing electrophoretic mobility.

in Table 5. In this analysis, the two sexual isolates present were ignored (T1 and T2 in Table 4). There is significant linkage disequilibrium between the PGI and PGM loci and between the PGM and SOD loci. This disequilibrium is a striking feature of the genetic structure of the population.

DISCUSSION

Considerable variation in phenotypic diversity for virulence was found within the five natural populations of *C. gloeosporioides* collected from *S. guianensis* (Tables 2 and 3). In contrast, isozymic diversity was strictly limited. Single multilocus isozyme phenotypes were detected in the four smaller populations (Calzada, Lamas, Quilichao, and Tarapoto). In three of these, the same multilocus phenotype was present (T5, Table 4), whereas the fourth population, Calzada, was fixed for different alleles at the PGM and SOD loci. Even in the more extensively sampled Darien population where 22 different virulence phenotypes were detected, electrophoretic variation was limited. A total of six multilocus isozyme phenotypes were found in this population. Three of these were relatively common (Table 4). However, the remaining three were each detected only once; two of these phenotypes were associated with teleomorphic isolates. Such marked differences in the level of diversity detected using racial and enzymatic analyses have been found previously in studies of *Puccinia recondita* Roberge f. sp. *tritici* (Eriksson) C. O. Johnston (2,5) and *P. striiformis* Westend. (16) and in some (2) but not all studies (6) of *P. graminis* Pers. f. sp. *tritici* Eriks. & E. Henn.

Comparisons of virulence and multilocus isozyme phenotypes in the one population in which both these characters varied (Darien) failed to detect any association. Thus, examples of the most and least virulent pathogen isolates (for example, virulence phenotypes 2 and 7) had the same isozyme phenotype (Table 3). More importantly, however, 80% of the isolates with the same virulence phenotype had distinctly different multilocus isozyme phenotypes (for example, virulence phenotypes 8 and 9).

TABLE 5. X^2 tests for association between alleles at the PGI, PGM, and SOD loci in the Darien population[a]

Locus pair	X^2 (1)	P
PGI and PGM	14.19	<0.005
PGI and SOD	3.25	n.s.
PGM and SOD	4.79	<0.05

[a] The two sexual isolates occurring at this site are excluded from the analysis. Values given incorporate Yates' correction for small sample sizes (20).

Such a lack of association between virulence and isozyme characters has been observed previously in a sexually reproducing population of *P. g. tritici* (6). However, in that case, a large number of multilocus isozyme phenotypes was detected (80 in 92 isolates), indicating random recombination of alleles at different isozyme loci. In the current interaction, the evidence for significant linkage disequilibrium between alleles at the PGI-PGM and PGM-SOD loci in *C. gloeosporioides* (Table 5) is strongly indicative of a uniparental breeding system (inbreeding or clonal reproduction). Furthermore, although no specific studies have been made, observations and experience with *C. gloeosporioides* reinforce the finding that the amount of sexual recombination in these populations is very limited (17, J. M. Lenné, *personal observations*).

S. guianensis is a perennial plant on which the pathogen may survive as anamorphic lesions during the dry season. Certainly, *C. gloeosporioides* commonly forms the teleomorph *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk toward the end of the wet season, but these do not appear to develop through crossing with other isolates. Several attempts have been made to mate isolates in culture, but these never have been successful (J. M. Lenné, *unpublished data*).

The most convincing demonstration that the sexual reproduction was an important source of variation in populations of *C. gloeosporioides* would have been the detection, among sexual isolates, of novel isozyme phenotypes that represented a recombination of existing forms. In this respect, the rare multilocus isozyme phenotype T3 detected at Darien may have arisen as a result of recombination between phenotypes T5 and T7. However, the multilocus isozyme phenotypes of both teleomorphic isolates (T1 and T2) incorporate unique alleles at the PGI, PGM, and SOD loci. As a consequence, their origin and role in the Darien population remain unclear.

In many previously studied host-pathogen associations, resistance in the host and virulence in the pathogen are controlled by single genes with major phenotypic effects. In contrast, resistance to *C. gloeosporioides* in *S. guianensis*, and by inference presumably virulence in *C. gloeosporioides*, are usually quantitative in effect, being controlled by many genes (13). In such situations, studies of electrophoretic variation provide valuable information concerning the genetic background against which the diversity of virulence phenotypes, which presumably is under strong selective pressure, is expressed.

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