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Genetic diversity in South American *Colletotrichum gloeosporioides* isolates from *Stylosanthes guianensis*, a tropical forage legume

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Abstract

The degree of genetic diversity of 127 *Colletotrichum gloeosporioides* isolates from *Stylosanthes guianensis* genotypes in South America was measured at the molecular level by random amplified polymorphic DNA (RAPD) with nine arbitrary primers of 10 bases, and by restriction fragment length polymorphism (RFLP) with a non-LTR (long terminal repeats) retrotransposon DNA sequence. The RAPD products revealed scorable polymorphism among the isolates, and a total of 80 band positions were scored. Sixty-three of the 127 isolates were clustered into 13 distinct lineages usually correlating with geographic origin. Where isolates from various regions were clustered together, most had identical host genotype origin. The pathogen population sampled from Carimagua, Colombia, a long-time *Stylosanthes* breeding and selection site, with a savanna ecosystem, was highly diverse. A set of 12 *S. guianensis* genotype differentials was used to characterize pathogenic variability of 104 isolates and their virulence patterns were grouped into 57 pathotypes. However, when they were tested on four Australian differentials, they grouped into 11 pathotypes. As shown in previous studies, no strict correlations existed between genetic diversity measured by RAPD or RFLP, and pathotype defined by pathogenicity pattern on the differentials. Southern blot analysis of the 127 isolates revealed 23 hybridizing fragments, resulting in 41 fingerprint patterns among the 127 isolates. Relationships between RFLP and RAPD variables were examined using Spearman's Rank Correlation Coefficient, which showed that the two measures of genotypic variation are in agreement.

Introduction

Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Sacc., is an important disease of *Stylosanthes guianensis*, and of world-wide distribution (Lenné et al., 1984). The host is a diverse tropical and subtropical forage legume of great potential that is naturally distributed in Central and South America (Williams et al., 1984). Total dry matter losses and reduced nutritional value have been reported in Colombia (CIAT, 1981); a loss of up to 80% has been reported in *S. guianensis* in Africa (Maraite, 1981, cited by Lenné and Calderon, 1984). Severe seed yield losses have been recorded in *S. guianensis* and other species of the *Stylosanthes* genus in Australia (Irwin and Cameron, 1978).

C. gloeosporioides is a heterogeneous and complex species, consisting of various host-specific populations. It exhibits extreme variability in both morphology (Cox and Irwin, 1988) and pathogenicity (Lenné

and Burdon, 1990). All virulent isolates from *Stylosanthes* spp. have been anamorphic (Ogle et al., 1986; Miles and Lenné, 1984). Irwin and Cameron (1978) described two anthracnose diseases of *Stylosanthes* caused by the pathogen in Australia. One, designated as Type A symptom, comprises a distinct anthracnose lesion, with a light-coloured centre and dark margins, and occurs in species of *Stylosanthes* other than *S. guianensis*. Type B symptom is a general necrosis, causing blight of terminal shoots and even plant death, and was described occurring on *S. guianensis* only.

The major centre of origin of the genus *Stylosanthes* (Williams et al., 1984), and thus the presumed center of genetic diversity of its pathogen, is in South America. Although understanding the pathogenic and genetic variability among isolates of the pathogen is key to creating effective breeding programs for anthracnose resistance and deployment of resistance, very little is known about the extent of such variability in South America. Miles and Lenné (1984) reported genetic variation for both morphological traits and pathogenicity among 16 isolates of *C. gloeosporioides* collected from a single site in Colombia. The pathogenicity of these 16 isolates was determined on 16 first-generation selfed progenies from *S. guianensis*. Lenné and Burdon (1990) observed considerable pathogenic variation among 69 isolates of *C. gloeosporioides* collected in five natural stands of *S. guianensis* in South America. One limiting factor in the study of pathogenic variability in the South American pathogen population had been the lack of appropriate differential hosts, now described by Kelemu et al. (1996).

To assess the genetic diversity among isolates of *C. gloeosporioides* in South America, we used RAPD, a technique that has been used not only for pathogen detection (Schesser et al., 1991), but also for race or species differentiation (Crowhurst et al., 1991; Goodwin and Annis, 1991; Jones and Dunkle, 1993; Schilling et al., 1996) and population genetics (McDonald and McDermott, 1993; Folkertsma et al., 1994).

DNA restriction fragment length polymorphisms detect variation in DNA sequences among homologous sections of chromosomes. Several studies of pathogenic fungi have been reported on RFLPs in nuclear DNA (Levy et al., 1991; Goodwin et al., 1992). He et al. (1996) isolated a repetitive element, termed CgT1 (*Colletotrichum gloeosporioides* Transposon 1), which is dispersed in the genome of *C. gloeosporioides* and present in about 30 copies. The CgT1 DNA probe detected polymorphism in Type B anthracnose-causing isolates of the fungus specific to *S. guianensis* (He et al., 1996). In this study, we used the CgT1 DNA probe to analyze the complexity of the pathogen population in South America. The molecular analysis of *C. gloeosporioides* isolates infecting *Stylosanthes* has been reviewed extensively by Manners et al. (1992).

The objectives of this study were to determine the variability in pathogenicity of South American isolates of *C. gloeosporioides*, to measure the degree of genetic diversity by RAPD and RFLP analysis, and also to evaluate possible correlations between pathogenicity and genetic diversity.

Materials and methods

Fungal isolates

Except for three Australian isolates, all isolates of *C. gloeosporioides* used in this study were collected in various regions of South America from naturally infected *S. guianensis* plants (Table 1). These included the 45 isolates (coded P) previously described by Kelemu et al. (1996, 1997). Isolates were obtained from diseased leaves, flowers, or stems of accessions of *S. guianensis*, and monoconidial cultures of these isolates were derived as described in Kelemu et al. (1966). Isolates collected before 1992 were chosen from collections maintained at the Centro Internacional de Agricultura Tropical (CIAT), Colombia.

Plant materials

Twelve differential *S. guianensis* genotypes described by Kelemu et al. (1996) were used to differentiate pathotypes. These included six new, single-seed-descent, inbred lines of *S. guianensis*, four genotypes used as differentials in Australia (cultivars Cook, Endeavour, Graham, and CPI 18750), Mineirão (a Brazilian cultivar released as anthracnose resistant), and accession CIAT 184 (a widely grown genotype in South America and Southeast Asia). Seeds were scarified with sandpaper and surface-sterilized in 1% NaOCl solution for 5 min. They were then rinsed three times with sterile deionized water and pregerminated on three layers of wet filtre paper. Five-day-old seedlings were transplanted to Jiffy pots (5.7×3.7 cm) containing 100 g of steam-sterilized Oxisol field soil from the CIAT Quilichao substation (Santander de Quilichao,

Table 1. Number, geographic origin, collection date, and lineages of the 127 *Colletotrichum gloeosporioides* isolates used in this study

Isolate CIAT no.	Host Acc. No.	Origin	Collection date	RAPD lineage	RFLP cluster	Pathotype
10188	1925	Carimagua, Colombia	04/01/80	7	1	42
10214	1684	Carimagua, Colombia	03/28/80	8	1	52
10309	18	Carimagua, Colombia	07/05/80	4	7	53
10501	1203	Carimagua, Colombia	07/06/81	7	7	3
10514	1581	Carimagua, Colombia	07/14/81	4	7	27
10518	1905	Carimagua, Colombia	07/18/80	3	7	55
10579	184	Pucallpa, Peru	06/03/81	x	1	4
10643 p	1391	Carimagua, Colombia	03/02/81	x	7	21
10676	1286	Carimagua, Colombia	02/20/81	7	1	2
10775	12	Carimagua, Colombia	07/24/81	x	1	51
10909 p	184	Quilichao, Colombia	08/29/81	x	1	7
10952	1508	Quilichao, Colombia	10/09/81	7	1	28
11006	10139	Quilichao, Colombia	11/19/81	7	1	39
11233	17	Barrolandia, Brazil	10/16/81	8	1	40
11372 p	184	Paragominas, Brazil	04/07/82	x	4	22
11378	184	Epamigsta, Brazil	03/15/82	1	1	53
11824	1175	Leticia, Colombia	11/09/82	2	7	55
11932 p	184	Pucallpa, Peru	02/08/83	x	1	12
11959	194	Pucallpa, Peru	03/13/83	7	1	50
11970	184	Yurimagua, Peru	02/24/83	7	4	7
12246	.	Tarapoto, Peru	08/04/83	1,2	1	55
12360	1950	Bahia, Brazil	08/27/83	2	7	55
12420	.	Boa Vista, Brazil	11/03/83	3	7	—
12613	1684	Yurimagua, Peru	12/12/ 83	x	8	56
12622 p	184	Pucallpa, Peru	05/23/83	1	3	19
13366 p	184	Carimagua, Colombia	05/25/85	x	7	18
13373 p	184	Carimagua, Colombia	05/25/85	x	7	16
13376	184	Carimagua, Colombia	05/25/85	x	1	35
13393 p	184	Carimagua, Colombia	10/31/84	1	7	23
14101 p	184	Carimagua, Colombia	05/28/87	12	1	20
15584	184	Carimagua, Colombia	03/28/94	x	7	43
16064 p	10941	Carimagua, Colombia	03/28/94	x	7	17
16065 p	11062	Carimagua, Colombia	03/16/94	1	7	23
16067	10417	Planaltina, Brazil	03/16/94	x	6	34
16068	2976	Planaltina, Brazil	03/16/94	1,2	2	54
16070	Bandeira	Planaltina, Brazil	03/16/94	1,2	2	57
16076	Mineirão	Planaltina, Brazil	03/16/94	x	5	24
16081	184	Caquetá, Colombia	03/16/94	x	1	7
16090	184	Caquetá, Colombia	04/01/94	x	1	33
16091	184	Caquetá, Colombia	04/01/94	7	1	4
16093 p	1280	Caquetá, Colombia	04/01/94	x	6	5
16094 p	1280	Caquetá, Colombia	04/01/94	x	6	4
16095	1280	Caquetá, Colombia	04/01/94	x	6	1
16105	11760	Carimagua, Colombia	04/28/94	1	7	47
16106	11753	Carimagua, Colombia	04/28/94	x	7	30
16110	10485	Planaltina, Brazil	03/16/94	x	1	—
16112 p	184	Caquetá, Colombia	04/01/94	11	1	14
16113 p	184	Caquetá, Colombia	04/01/94	13	1	4
16114 p	184	Caquetá, Colombia	04/01/94	x	1	3
16117	184	Caquetá, Colombia	04/01/94	10	1	2
16118 p	184	Caquetá, Colombia	04/01/94	x	1	4
16119 p	184	Caquetá, Colombia	04/01/94	x	1	2

Table 1. (continued)

Isolate CIAT no.	Host Acc. No.	Origin	Collection date	RAPD lineage	RFLP cluster	Pathotype
16122 p	184	Caquetá, Colombia	04/01/94	10	1	10
16123	184	Caquetá, Colombia	04/01/94	10	1	29
16124 p	184	Caquetá, Colombia	04/01/94	10	1	4
16125 p	184	Caquetá, Colombia	04/01/94	12	1	4
16128 p	184	Caquetá, Colombia	04/01/94	x	1	10
16131 p	184	Caquetá, Colombia	04/01/94	x	1	13
16132 p	184	Caquetá, Colombia	04/01/94	x	1	2
16133 p	184	Caquetá, Colombia	04/01/94	x	1	6
16134 p	184	Caquetá, Colombia	04/01/94	10,11	1	1
16135 p	184	Caquetá, Colombia	04/01/94	10	1	3
16136	184	Caquetá, Colombia	04/01/94	x	1	4
16137 p	184	Caquetá, Colombia	04/01/94	12	1	11
16139	184	Caquetá, Colombia	04/01/94	10	1	3
16140 p	184	Caquetá, Colombia	04/01/94	10	1	4
16141 p	184	Caquetá, Colombia	04/01/94	x	1	8
16142	184	Caquetá, Colombia	04/01/94	x	1	4
16143	184	Caquetá, Colombia	04/01/94	10	1	—
16144	184	Caquetá, Colombia	04/01/94	10	1	38
16145 p	184	Caquetá, Colombia	04/01/94	13	1	7
16146 p	184	Caquetá, Colombia	04/01/94	10	1	7
16147 p	184	Caquetá, Colombia	04/01/94	x	1	7
16151	184	Caquetá, Colombia	04/01/94	x	1	1
16162 p	184	Caquetá, Colombia	04/01/94	10	1	4
16166 p	184	Caquetá, Colombia	04/01/94	x	1	2
16168	184	Caquetá, Colombia	04/01/94	x	1	—
16170	184	Caquetá, Colombia	04/01/94	x	1	26
16172 p	184	Caquetá, Colombia	04/01/94	x	1	9
16173 p	184	Caquetá, Colombia	04/01/94	x	1	9
16176 p	184	Caquetá, Colombia	04/01/94	x	1	4
16179 p	184	Caquetá, Colombia	04/01/94	x	1	7
16181 p	184	Caquetá, Colombia	04/01/94	x	1	10
16182 p	184	Caquetá, Colombia	04/01/94	x	1	4
16191 p	184	Carimagua, Colombia	06/16/94	x	1	15
16192 p	184	Carimagua, Colombia	06/16/94	x	1	4
16193	FM9205P2	Carimagua, Colombia	06/16/94	1	3	48
16194	FM9205P2	Carimagua, Colombia	06/16/94	x	3	37
16196	FM9205P6	Carimagua, Colombia	06/16/94	x	1	31
16197 p	184	Carimagua, Colombia	06/16/94	x	1	4
16198	FM0186P6	Carimagua, Colombia	06/16/94	5	7	31,41
16200	184	Carimagua, Colombia	06/16/94	x	1	7
16201	FM23	Carimagua, Colombia	06/16/94	x	4	25
16202 p	FM0186P6	Carimagua, Colombia	06/16/94	x	7	23
16204	FM9205P6	Carimagua, Colombia	06/16/94	1	4	3
16205	FM9205P2	Carimagua, Colombia	06/16/94	1	4	32
16207	FM9205P3	Carimagua, Colombia	06/16/94	x	7	45
16208	184	Carimagua, Colombia	06/16/94	5	7	57
16209	FM9205P2	Carimagua, Colombia	06/16/94	1	3	49
16215	FM9205P3	Carimagua, Colombia	06/16/94	x	4	36
16216	136	Carimagua, Colombia	06/16/94	x	1	3
16218	FM9205P2	Carimagua, Colombia	06/16/94	1	4	57
16261	Mineirão	Campo Grande, Brazil	—	9	5	—
16264	Mineirão	Campo Grande, Brazil	—	x	5	—
16266	Mineirão	Campo Grande, Brazil	—	9	5	—

Table 1. (continued)

Isolate CIAT no.	Host Acc. No.	Origin	Collection date	RAPD lineage	RFLP cluster	Pathotype
16269	.	Planaltina, Brazil	—	x	1	—
16270	.	Planaltina, Brazil	—	x	1	—
16276	11844/33	Quilichao, Colombia	06/28/94	6	9	—
16280	11844/33	Quilichao, Colombia	06/28/94	x	9	—
16281	11844/33	Quilichao, Colombia	06/28/94	6	9	—
16283	11844/33	Quilichao, Colombia	06/28/94	6	9	—
16284	11844/33	Quilichao, Colombia	06/28/94	x	9	—
16288	11844/33	Quilichao, Colombia	06/28/94	6	9	—
16289	11844/33	Quilichao, Colombia	06/28/94	6	9	—
16293	11844/33	Quilichao, Colombia	06/28/94	6	7	—
16294	11844/33	Quilichao, Colombia	06/28/94	6	9	—
16295	11844/33	Quilichao, Colombia	06/28/94	1	9	—
16320	Mineirão	Campo Grande, Brazil	—	9	5	—
16328	Mineirão	Campo Grande, Brazil	—	x	7	—
16342	.	Campo Grande, Brazil	—	9	1	—
16343	.	Campo Grande, Brazil	—	9	1	—
16361	.	Minas Gerais, Brazil	—	x	5	—
16362	BRA17817	Campo Grande, Brazil	—	x	7	—
16363	BRA17817	Campo Grande, Brazil	—	9	5	—
UQ27	.	Australia	—	x	1	44
UQ62	.	Australia	—	x	1	46
WRS36	.	Australia	—	x	1	57

x – RAPD lineage not clearly determined; note that isolates 12246, 16068, 16070, 16134 each fall into two different RAPD lineages.

Department of Cauca, Colombia) and supplemented with N–P–K fertilizer (15–15–15) at a rate of 3.6 g kg^{-1} of soil. The plants were grown in a glasshouse with natural daylight and with temperatures between 19 and 30 °C.

Pathogenicity and disease ratings

A total of 104 isolates of *C. gloeosporioides* were tested on the differential host genotypes. Inoculum preparation, inoculations, and disease evaluations were conducted as described by Kelemu et al. (1996). Disease evaluations were arranged in a split-plot design with each run laid out as a randomized block with three replicates. Each replicate contained five to eight plants. Isolates CIAT 16118, 16125, 16134 (arbitrarily chosen), and Australian isolates WRS36, UQ27, and UQ62 were included as references for each set of inoculations. Virulence tests were repeated at least once for verification.

DNA isolations and RFLP analyses

For DNA isolations, fungal cultures were grown in fresh V-8 juice broth for 3 days at 28 °C on a shaker at 200 rpm. DNA was isolated using the method of Braithwaite et al. (1990). Genomic DNA (3–5 μg) from each isolate was digested to completion with *Eco*RI at 37 °C, as recommended by the enzyme manufacturer (Gibco BRL). DNA fragments were separated by electrophoresis in 0.8% agarose gels in 1× TBE buffer (Sambrook et al., 1989) at 33 V for 48 h and blotted on to Hybond N+ membrane (Amersham) by alkali transfer, as described by the manufacturer.

An ECL direct nucleic acid detection system kit (Amersham) was used as directed by the manufacturer to detect hybridized bands and label the pCHB1 probe containing a repetitive element (He et al., 1996).

DNA amplification conditions

Nine, arbitrary 10-base, oligonucleotide primers from Operon Technologies [5′-TGCCGAGCTG-3′ (primer code A-02), 5′-AGTCAGCCAC-3′ (A-03), 5′-GTCGGAGTGG-3′ (AJ-06), 5′-ACGGCACGCA-3′ (AJ-09), 5′-AGGGTCGGTC-3′ (AK-04), 5′-AGGTCGGCGT-3′ (AK-09), 5′-TCGCAGCGAG-3′ (AK-19), 5′-GTGAGGCGTC-3′ (C-02), and

5′-GTCGCCGTCA-3′, (D-03)] were used for PCR amplification. PCR reactions (25 μl) contained pre-prepared buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) from Perkin Elmer (USA), 200 μm each dNTP, 0.5 μm primer, 1 unit of Taq DNA Polymerase, 2.5 mM $MgCl_2$, and 20 ng DNA template. Amplifications were performed in a Programmable Thermal Controller (MJ Research, Inc.), programmed with 45 cycles of a 1-min (2 min for the first cycle) denaturation step at 94 °C, annealing for 1 min at 35 °C, and primer extension for 1 min (7 min in the final cycle) at 72 °C. The amplification products were resolved by electrophoresis in a 1.2% agarose gel (Bio-Rad), stained with ethidium bromide, and photographed under UV lighting (e.g., Figure 1).

Analysis of RAPD data

Comparisons of each banding profile for each primer were conducted on the basis of presence or absence (1/0) of RAPD products of the same size. Bands of the same size were scored as identical. An analysis of variation over the entire data set was undertaken to assess the probable number of genetic lineages present. First, coefficients of dissimilarity (1-Jaccard's coefficient) were calculated for all combinations of isolates, using the SAS-PROC IML program (1989a). A multiple correspondence analysis (MCA) was carried out on the distance data, using PROC CORRESP (SAS, 1989b). A cluster analysis was carried out, using PROC CLUSTER (SAS, 1989b), with several clustering methods and parameters. The clustering statistics cubic clustering criterion (CCC), pseudo F, and pseudo t^2 were graphed. A consensus of the three statistics [local maxima of CCC and pseudo F concomitant with a local minimum of pseudo t^2 (SAS, 1989b, No. 11, page 98)] was sought as an indication of the number of modal clusters present in the data set. Consensus of the various clustering methods on the number of clusters present was accepted as an indication of the number of distinct genetic lineages represented in the data set.

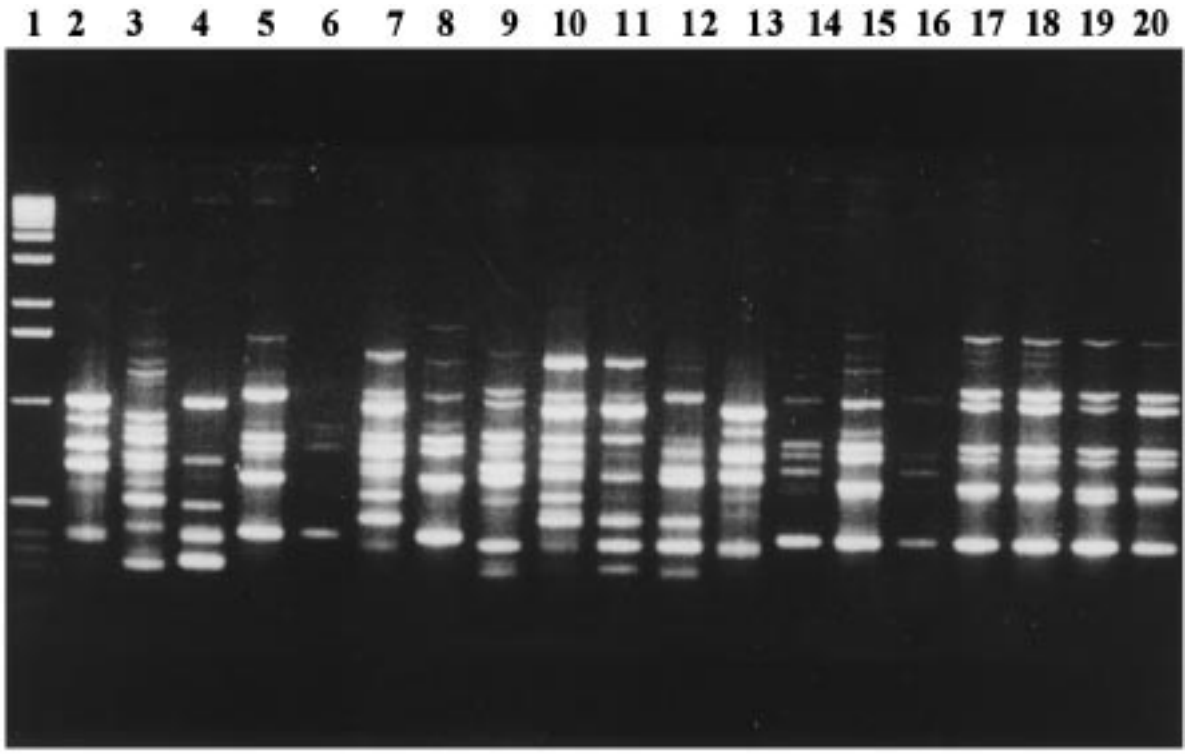

Figure 1. Examples of DNA from isolates of *Colletotrichum gloeosporioides* amplified with a single arbitrary primer (AK-09). Isolates by lane number are: 1, marker 1 kb ladder; 2, CIAT 16201; 3, CIAT 15584; 4, CIAT 12622; 5, CIAT 16132; 6, CIAT 16192; 7, CIAT 16194; 8, CIAT 16197; 9, CIAT 16198; 10, CIAT 16204; 11, CIAT 16193; 12, CIAT 16200; 13, CIAT 16205; 14, CIAT 10643; 15, CIAT 10775; 16, CIAT 16113; 17, CIAT 16114; 18, CIAT 16119; 19, CIAT 16118; 20, CIAT 16117.

A three-dimensional graph of the first three dimensions of the MCA analysis was constructed, using the 'spin' platform of JMP software (SAS, 1995) to provide a visual representation of the associations.

The next step, to test the statistical reproducibility of the associations found, was carried out by combining cluster analyses with the resampling ('bootstrapping') technique (Efron and Gong, 1983). The resampling analysis consisted of constructing 127×80 data sets by random sampling, with replacement from the original data set, then carrying out the cluster analysis, using the TWOSTAGE method with $K = 4$ on the new data set. The cluster assignments of each isolate were tabulated; the process was repeated 1000 times. 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 distinctive genetic lineage. The total number of lineages represented was estimated from the number of unique groups formed by this 90% criterion. This criterion assumes that RAPD fragments judged as being of the same size had a single historical origin, and that the clustering method used was sufficient to detect unique lineages.

RFLP data were analyzed using Ward's method (Ward, 1963). Correlations among RAPD lineage, RFLP, and pathotype were measured with Spearman's Rank Correlation Coefficients (Snedecor and Cochran, 1967).

Results

Pathogenicity and disease response

Disease symptoms (blight on leaves and stems) differed markedly between resistant and susceptible genotypes. Some isolates from the early 1980s were less virulent and may have lost virulence during culturing and storage (Kelemu et al., 1996). *Stylosanthes guianensis*-specific isolates (Type B-like isolates) CIAT 16093, 16094, 16133, 16134, 16135, 16140, 16162, 16176, 13373, 13376, and 16067 infected the supposedly resistant and nonhost *S. scabra* cv. Fitzroy (Kelemu et al., 1996), indicating that this group of South American isolates may represent a third biotype in addition to the two biotypes described in Australian studies. *S. guianensis* cv. Endeavour, used in Australia as the all-susceptible host, expressed resistant reactions to some South American isolates. This indicates that the South American pathogen population is more diverse in its composition.

Using 12 *S. guianensis* genotypes, including four Australian differentials, the 104 isolates tested grouped into 57 pathotypes. However, using only the four Australian differentials, these same isolates grouped into just 11 pathotypes. These results confirm findings of previous studies that showed that the differentials currently used in Australia are not sufficient to classify the complex South American pathogen population.

Statistical RAPD data analysis

Among the 127 isolates, 63 comprised 13 distinctive lineages by the 90% criterion. Four isolates were assigned to two lineages (isolates CIAT 12246, 16068, and 16070 in lineages 1 and 2; CIAT 16134 in lineages 10 and 11), indicating an overlap of lineages, and implying that one had diverged from the others. We define a lineage as a group of isolates that are assigned to the same cluster in more than or equal to 90% of the resampling iterations. None of the remaining 64 isolates (marked x in Table 1) clustered with any other isolate in more than 90% of the resampling iterations, suggesting that each of the 64 isolates were either unique, or were intermediate between clusters in RAPD fragment profiles and could have been part of more than one cluster with more-or-less equal likelihood.

The clusters defined by the 90% criterion are diagrammed in Figure 2. Cluster 1 consisted of 14 isolates. Eight of the 14 isolates had been collected in Carimagua, Colombia; the remaining six were from Brazil, Peru, or Colombia (Quilichao). Two Brazilian isolates and one from Peru 'overlapped' with cluster 2 (Figure 2), which contained an additional isolate from Brazil, and one from Leticia, Colombia.

Cluster 6 consisted of seven isolates that were distinct from all other isolates (Figure 2); all had been collected from Santander de Quilichao, Colombia, on June 28, 1994. Interestingly, these isolates were collected from a field planted with a mixture of two highly anthracnose-resistant hybrids (CIAT 11844 and 11833, or designated 11844/33 in Table 1). Cluster 9 consisted of six isolates from Campo Grande, Brazil.

Cluster 7 was comprised of eight isolates collected from a wide range of geographic sites and collection dates, while cluster 10 consisted of 12 isolates, all collected from the same site near Caquetá, Colombia, on April 1, 1994. An additional six isolates, collected from the same site and on the same date, formed three closely related clusters, each consisting of two isolates. One

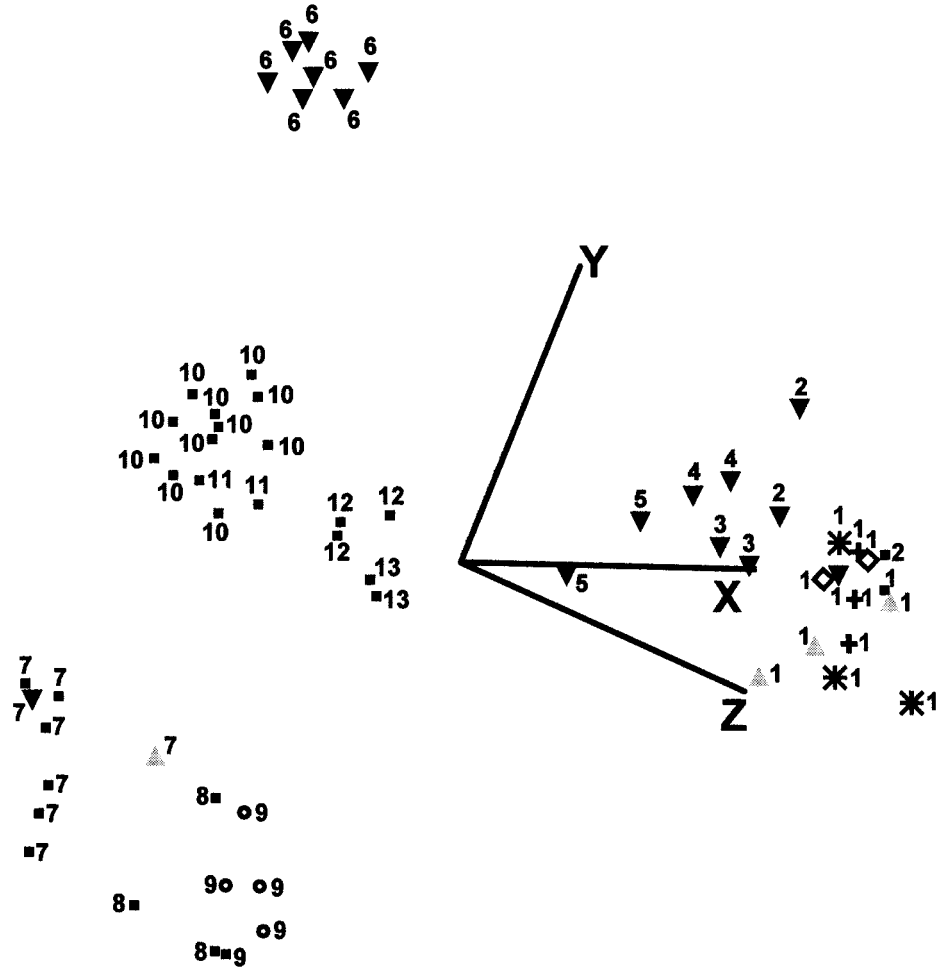

Figure 2. Sixty-three isolates of *Colletotrichum gloeosporioides* clustered by the 90% resampling iterations of RAPD data. Symbols represent RFLP clusters and numbers are RAPD lineages

of these, from Carimagua, and collected in 1987, also clustered with two of the isolates from Caquetá (cluster 12, Table 1). Interestingly, all the isolates in cluster 12, although collected from two locations 7 years apart, came from the same host genotype CIAT 184.

To analyze the genetic constitution of a *C. gloeosporioides* population, we chose a specific field of *S. guianensis* accession CIAT 184, in Caquetá, Colombia, April 1994, to interpret results. Among 41 isolates analyzed, 33 comprised an essentially homogeneous cluster with genetic distance less than 0.30. Three isolates, CIAT 16081, 16090, and 16091, were highly similar to each other with average genetic distances of 0.05, 0.15, and 0.20, respectively, but with an average genetic distance to the cluster of 33 isolates of 0.51. The remaining five isolates, CIAT 16119, 16136, 16147, 16168, and 16170, were each distinct from the other 36 isolates, showing genetic distances from any other isolate of 0.30 or more, and reaching a maximum of 0.74. Average distances to the other 36 isolates for CIAT 16119, 16136, 16147, 16168, and 16170 were 0.54, 0.40, 0.43, 0.48, and 0.49, respectively.

At Santander de Quilichao, in June 1994, 10 isolates were collected from a field of mixed *S. guianensis* improved hybrids (CIAT 11844 and 11833) developed to be resistant to anthracnose. Eight of the 10 were closely related, with genetic distances ranging from 0.09 to 0.42 and averaging 0.26. The remaining two isolates (CIAT 16280 and 16295) were distinct from the other eight (average distances were 0.75 and 0.80,

respectively), and from each other (genetic distance was 0.83). Thus, three distinct lineages apparently were collected from the Quilichao site.

Five isolates were collected from the anthracnose-resistant *S. guianensis* cv. Mineirão from Campo Grande, Brazil. Two additional isolates were collected from Campo Grande from an unspecified cultivar. Of these seven isolates, five (CIAT 16261, 16266, 16320, 16342, and 16343) were closely related with a maximum genetic distance of 0.27 and a mean of 0.18; the other two isolates (CIAT 16264 and 16328) were distinct from the first five (mean genetic distances of 0.38 and 0.69, respectively) and to each other (genetic distance of 0.69). This result was similar to those for the collections from Quilichao and Caquetá.

Eighteen isolates were collected in June 1994 from *S. guianensis* on various accessions grown at Carimagua. This is a surprisingly diverse collection of isolates. The most similar isolates (CIAT 16216 and 16197) had a genetic distance of 0.34. All other pairs of *C. gloeosporioides* isolates in this collection differed by genetic distances ranging from 0.47 to 0.94, with an overall mean of 0.77.

Eighteen additional isolates had been collected previously from the Carimagua site as early as March 1980. In general, this collection of 36 *C. gloeosporioides* isolates (collected as early as March 1980 and June 1994) is extremely diverse. A highly different isolate of *C. gloeosporioides* (CIAT 16065) was cultured from *S. guianensis* accession CIAT 11062 at Carimagua in March 1994, showing a minimum genetic distance from all other Carimagua isolates of 0.74, a maximum of 0.95, and a mean of 0.83. All 36 isolates from Carimagua had genetic distances that ranged from 0.22 to 1.0, with an overall mean of 0.74.

Among the isolates collected from host accession CIAT 184, five isolates were distinct with genetic distances from 0.47 to 0.94. Thus, host genotype apparently had no influence on the genetic constitution of those five isolates.

RFLP data

RFLP analysis of the 127 isolates, with pCHB1 as a probe, revealed a low level of variation (e.g., Figure 3). Twenty-three hybridizing fragments were found, resulting in 41 fingerprint patterns among the isolates. Clustering of the RFLP fingerprint patterns divided the 127 isolates into nine groups using Ward's minimum variance method (Table 1). Twenty-three

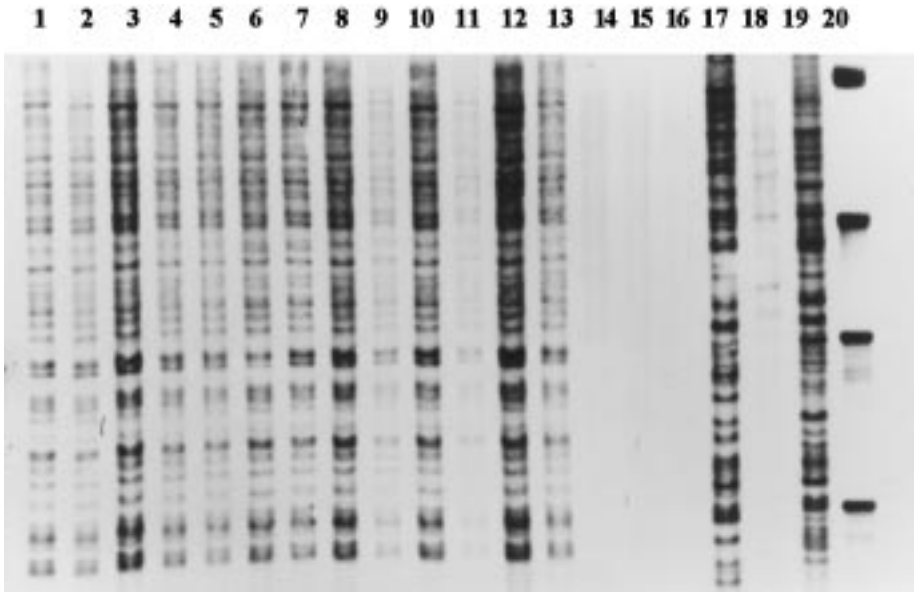

Figure 3. Examples of Southern hybridization of probe CgT1 to a gel blot of genomic DNA of isolates of *Colletotrichum gloeosporioides* digested with *Eco*RI. Isolates by lane number are: 1, CIAT 16112; 2, CIAT 16118; 3, CIAT 16122; 4, CIAT 16134; 5, CIAT 16137; 6, CIAT 16145; 7, CIAT 16146; 8, CIAT 16162; 9, CIAT 16166; 10, CIAT 16172; 11, CIAT 16173; 12, CIAT 16181; 13, CIAT 16182; 14, CIAT 16202; 15, CIAT 16064; 16, CIAT 10643; 17, CIAT 10909; 18, CIAT 12622; 19, CIAT 14101; 20, λ HindIII marker.

isolates had no fragments that hybridized to the probe, indicating a lack of the repetitive element. Among these 23 isolates, six were from various locations, and 17 were isolated from Carimagua, collected over a period of 14 years from 1980 to 1994. This result indicated that a population of the fungus, lacking the repetitive element, persisted in the area for years. Five of these isolates were collected on June 16, 1994. However, another six isolates from Carimagua, collected on that same day, all carried the pCHB1 element, hybridizing to identical patterns that consisted of 19 of the 23 fragments. This is clear evidence of a second introduction of the fungus into the Carimagua area. RFLP analysis revealed 14 fingerprint patterns at Carimagua, confirming the presence of wide genetic variability.

Of 44 isolates collected in Caquetá, on April 1, 1994, 41 had identical pCHB1 patterns, hybridizing to 18 of the 23 fragments (part of RFLP cluster 1, Table 1). The remaining three had identical patterns, hybridizing to 13 of the total fragments. Interestingly, the 41 isolates were collected on host accession CIAT 184, whereas the three isolates with a different fingerprint came from host accession CIAT 1280.

Nine of the 10 isolates collected from the anthracnose-resistant hybrid mixture 11844/11833 in Quilichao on June 28, 1994 had identical pCHB1 patterns, hybridizing to two of the 23 fragments (RFLP cluster 9, Table 1). The tenth isolate, CIAT 16293, appeared to lack the repetitive sequence.

Four of the six isolates collected from the anthracnose-resistant Brazilian cultivar Mineirão had identical patterns, hybridizing to four of the fragments. Three Australian isolates included as test isolates all had identical hybridizing patterns.

Correlations of RAPD lineage, RFLP fingerprint patterns and pathotypes measured with Spearman's Rank Correlation Coefficients are presented in Table 2. The significant relatedness of RAPD and RFLP variables shows that these measures of genotypic variation are in agreement.

Table 2. Correlations between RFLP, RAPD and pathotype variables for isolates of *Colletotrichum gloeosporioides* using Spearman's Rank Correlation Coefficients

Variable 1	Variable 2	Spearman's 'r'	*P*
RAPD	RFLP	0.73	< 0.0001
RFLP	Pathotype	0.59	< 0.0001
RAPD	Pathotype	0.58	< 0.0001

Discussion

In this study, we examined pathotypes, RAPD and RFLP polymorphisms to evaluate genetic relatedness and diversity in an attempt to understand the South American *Colletotrichum gloeosporioides* population. We showed that those South American isolates of *C. gloeosporioides* that infect *S. guianensis* exhibit a wide range of genetic diversity. RAPD data analysis grouped 63 of the 127 isolates examined into 13 putative genetic lineages. Pathogenic variation, based on *S. guianensis* differentials, revealed that a wide range of pathotypes existed among the 104 isolates tested. New virulence patterns were detected among these isolates. The results of this study confirm previous observations that South America contains a diverse population of *C. gloeosporioides* (Kelemu et al., 1995, 1996, 1997).

We concluded that most of the population in Caquetá (33 of 41 isolates, 80%) were of the same genetic lineage, but that diversification occurred to the extent that the genetic distances among the 33 isolates averaged 0.27. Three isolates, CIAT 16081, 16090, and 16091, belonged to a lineage different to that of the other isolates, and five isolates each belonged to a unique lineage. Therefore, the *C. gloeosporioides* population in the field under study probably was derived from at least seven lineages, with considerable diversification of the endemic population. However, the population sample was only of isolates capable of causing lesions on *S. guianensis* accession CIAT 184. Additional genotypes were probably present, but not collected because they were not compatible with this host genotype.

RAPD clusters 6 and 9 are particularly interesting. Cluster 6 contained seven isolates, which were different from all other isolates (Table 1). These isolates were collected from Santander de Quilichao, from a grazing field planted with a mixture of new anthracnose-resistant hybrids (CIAT 11833 and 11844). The anthracnose lesions on the leaves of these hybrids comprised a consistent pattern of tiny spots. These isolates probably represent new variants not observed previously. Unfortunately, these isolates were not tested for their pathogenicity pattern on the differentials because seed was unavailable for some of the differentials. Cluster 9 consisted of six isolates collected from Campo Grande, Brazil, all except one, from the anthracnose-resistant Brazilian cultivar Mineirão released recently. The *C. gloeosporioides* collection from the Campo Grande site was apparently homogeneous, with occasional new genotypes introduced.

Determining the number of distinct genetic lineages in the collection of 36 Carimagua isolates was difficult, but the genetic diversification observed is unlikely to have derived from a single introduction of the fungus. This may indicate that many *C. gloeosporioides* genotypes are endemic to this area.

In summary, the genetic situation of *C. gloeosporioides* populations that can cause anthracnose in *S. guianensis* appears to be as follows. Most sites are populated by fairly homogeneous endemic populations that have undergone diversification to some degree, which, however, was usually evidenced as less than 30% difference by RAPD fragment profiles. A substantial number of isolates, about 20% of a site's population, are very different from the bulk of that population, suggesting that multiple introductions to the site may have occurred. This kind of pattern was evident in the Campo Grande, Quilichao, and Caquetá sites.

For Carimagua, the population sampled was highly diverse, with few isolates shown as related at more than 40% similarity in RAPD fragment profiles. However, Carimagua has been under *S. guianensis* cultivation longer than the other sites sampled, thus allowing time for more *C. gloeosporioides* diversification. Alternatively, numerous *C. gloeosporioides* introductions may have occurred in that area.

No strict correlation was observed between pathogenicity pattern on *S. guianensis* differentials and RAPD or RFLP data, although Spearman's correlation coefficients indicted that there is significant relatedness among the three measures. This apparent lack of strict correlation is not necessarily surprising; if the amount of genetic diversity within a pathotype is very large relative to the magnitude of variability among the various pathotypes, we then expect the differences that distinguish the pathotypes to be diminished. In other words, the degree to which RAPD polymorphisms can differentiate pathotypes into distinct groups depends on there being heterogeneity among pathotypes, supplemented with enough homogeneity among isolates within a pathotype (Kelemu et al., 1997). Our results are consistent with the findings of others that isolates of the same race are not necessarily closely related (Jacobson and Gordon, 1990; Woo et al., 1996). Goodwin et al. (1995) reported pathotype diversity within lineages in *Phytophthora infestans*. Evidently, pathogenicity evolved independently of molecular markers within clonal lineages in potato/*Phytophthora infestans* interactions. On the other hand, Levy et al. (1993), using DNA fingerprint analysis, showed that each clonal lineage of the rice blast fungus *Magnaporthe grisea* was associated with a particular subset of pathotypes. A general correspondence between isolate groups and their geographic origin was observed.

The absence of CgT1 in some of the isolates tested agrees with the findings reported by He et al. (1996), in that this element appears to have a restricted distribution within the group species *C. gloeosporioides*. This discontinuous distribution means that DNA hybridizations using CgT1 or probes derived from it may not be useful to determine clonality among isolates of *C. gloeosporioides*.

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References

Braithwaite KS, Irwin JAG and Manners JM (1990) Restriction fragment length polymorphisms in *Colletotrichum gloeosporioides* infecting *Stylosanthes* spp. in Australia. Mycol Res 94: 1129–1137

CIAT (Centro Internacional de Agricultura Tropical) (1981) Annual Report 1981. CIAT, Cali (Colombia)

Cox ML and Irwin JAG (1988) Conidium and appressorium variation in Australian isolates of the *Colletotrichum gloeosporioides* group and closely related species. Aust Syst Bot 1: 139–149

Crowhurst RN, Hawthorne BT, Rikkerink EHA and Templeton MD (1991) Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Curr Genet 20: 391–396

Efron G and Gong F (1983) A leisurely look at the bootstrap, the jackknife and cross-validation. Am Stat 37: 35–48

Folkertsma RT, Rouppe van der Voort JNAM, van Gent-Pelzer MPE, de Groot KE, van den Bos WJ, Schots A, Bakker J and Gommers FL (1994) Inter- and intraspecific variation between populations of *Globodera rostochiensis* and *G. pallida* revealed by random amplified polymorphic DNA. Phytopathology 84: 807–811

Goodwin PH and Annis SL (1991) Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Appl Environ Microbiol 57: 2482–2486

Goodwn SB, Drenth A and Fry WE (1992) Cloning and genetic analyses of two highly polymorphic, moderately repetitive nuclear DNAs from *Phytophthora infestans*. Curr Genet 22: 107–115

Goodwin SB, Sujkowski LS and Fry WE (1995) Rapid evolution of pathogenicity withinclonal lineages of the potato late blight disease fungus. Phytopathology 85: 669–676

He C, Nourse JP, Kelemu S, Irwin JAG and Manners JM (1996) CgT1: a non-LTR retrotransposon with restricted distribution in the fungal phytopathogen *Colletotrichum gloeosporioides*. Mol Gen Genet 252: 320–331

Irwin JAG and Cameron DF (1978) Two diseases of *Stylosanthes* spp. caused by *Colletotrichum gloeosporioides* in Australia and pathogenic specialization within one of the causal organisms. Aust J Agric Res 29: 305–317

Jacobson DJ and Gordon TR (1990) Variability of mitochondrial DNA as an indicator of relationships between populations of *Fusarium oxysporum* f. sp. *melonis*. Mycol Res 94: 734–744

Jones MJ and Dunkle LD (1993) Analysis of *Cochliobolus carbonum* races by PCR amplification with arbitrary and gene-specific primers. Phytopathology 83: 366–370

Kelemu S, Badel JL, Moreno CX and Miles JW (1996) Virulence spectrum of South American isolates of *Colletotrichum gloeosporioides* on selected *Stylosanthes guianensis* genotypes. Plant Dis 80: 1355–1358

Kelemu S, Badel JL, Moreno CX, Miles JW, Chakraborty S, Fernandes CD and Charchar MJ (1997) Biodiversity, epidemiology and virulence of *Colletotrichum gloeosporioides*. I. Genetic and pathogenic diversity in *Colletotrichum gloeosporioides* isolates from *Stylosanthes guianensis*. Trop Grass 31: 387–392

Kelemu S, Moreno CX, Rodriguez MX and Badel JL (1995) Genetic diversity among isolates of *Colletotrichum gloeosporioides* infecting forage legume *Stylosanthes* spp. (Abstr.) Phytopathology 85: 1201

Lenné JM and Burdon JJ (1990) Preliminary study of virulence and isozymic variation in natural populations of *Colletotrichum gloeosporioides* from *Stylosanthes guianensis*. Phytopathology 80: 728–731

Lenné JM and Calderon MA (1984) Diseases and pest problems of *Stylosanthes*. In: Stace HM and Edye LA (eds.) The Biology and Agronomy of *Stylosanthes* (pp 279–293) Academic Press, Sydney, Australia

Lenné JM, Thomas D, de Andrade RP and Vargas A (1984) Anthracnose (*Colletotrichum gloeosporioides*) of *Stylosanthes capitata*: Implications for future disease evaluation of indigenous tropical pasture legumes. Phytopathology 74: 1070–1073

Levy M, Correa-Victoria FJ, Zeigler RS, Xu S and Hamer JE (1993) Genetic diversity of the rice blast fungus in a disease nursery in Colombia. Phytopathology 83: 1427–1433

Levy M, Romao J, Marchetti MA and Hamer JE (1991) DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3: 95–102

Manners JM, Masel A, Braithwaite KS and Irwin JAG (1992) Molecular analysis of *Colletotrichum gloeosporioides* pathogenic on the tropical pasture legume *Stylosanthes*. In: Bailey JA and Jeger MJ (eds.) *Colletotrichum*: Biology, Pathology and Control (pp 250–268) CAB International, Wallingford, UK

McDonald BA and McDermott JM (1993) Population genetics of plant pathogenic fungi. BioScience 43: 311–319

Miles JW and Lenné JM (1984) Genetic variation within a natural *Stylosanthes guianensis*, *Colletotrichum gloeosporioides* host-pathogen population. Aust J Agric Res 35: 211–218

Ogle HJ, Irwin JAG and Cameron DF (1986) Biology of *Colletotrichum gloeosporioides* isolates from tropical legumes. Australian Journal of Botany 34: 281–292

Sambrook J, Fritsch E and Maniatis T (1989) Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

SAS (1989a) SAS/IML Software: Usage and Reference, Version 6, SAS Institute Inc., Cary, North Carolina

SAS (1989b) SAS/STAT User's Guide, Version 6, SAS Institute Inc., Cary, North Carolina

SAS (1995) SAS/JMP Statistical Discovery Software, Version 3.1, SAS Institute Inc., Cary, North Carolina

Schesser K, Luder A and Henson JH (1991) Use of the polymerase chain reaction to detect the take-all fungus, *Gaeumannomyces graminis* in infected wheat plants. Appl Environ Microbiol 57: 553–556

Schilling AG, Moller EM and Geiger HH (1996) Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. Phytopathology 86: 515–522

Snedecor GW and Cochran WG (1967) Statistical Methods, 6th ed. Iowa State University Press, Ames, IA, USA. 593 pp

Ward JH (1963) Hierarchical grouping to optimize an objective function. Journal of the American Statistical Association 58: 236–244

Williams RJ, Reid R, Schultze-Kraft R, Sousa Costa NM and Thomas BD (1984) Natural distribution of *Stylosanthes*. In: Stace HM and Edye LA (eds.) The Biology and Agronomy of *Stylosanthes* (pp 73–102) Academic Press, Sydney, Australia

Woo SL, Zoina A, Del Sorbo G, Lorito M, Nanni B, Scala F and Noviello C (1996) Characterization of *Fusarium oxysporum* f. sp. *phaseoli* by pathogenic races, VCGs, RFLPs, and RAPD. Phytopathology 86: 966–973