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Virulence and molecular diversity within *Colletotrichum lindemuthianum* isolates from Andean and Mesoamerican bean varieties and regions

George S. Mahuku and Jhon Jaime Riascos
Centro Internacional de Agricultura Tropical (CIAT), A. A. 6713, Cali, Colombia, South America (Fax: +57 2 445 0073; E-mail: g.mahuku@cgiar.org)

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Abstract

Virulence on a standard set of 12 common bean differential varieties, DNA sequence of repetitive-elements (Rep-PCR) and random amplified microsatellites (RAMS) were used to assess the genetic variability of 200 *Colletotrichum lindemuthianum* isolates collected from Andean and Mesoamerican bean varieties and regions. High levels of pathotypic (90 pathotypes) and genetic diversity (0.97) were identified among 200 isolates, revealing that *C. lindemuthianum* is a highly diverse pathogen. Although a significant number of pathotypes were common to Andean and Mesoamerican regions, many more were only found in the Mesoamerican region. Cluster analysis of virulence and molecular data did not separate isolates into groups that were structured with common bean gene pools. No genetic differentiation ($G_{ST} = 0.03$) was apparent between Andean and Mesoamerican isolates of *C. lindemuthianum*. The diversity exhibited by *C. lindemuthianum* does not appear to cluster according to common bean gene pools, and the high diversity found in the Mesoamerican region seems to indicate that *C. lindemuthianum* originated and was disseminated from this region. Due to the high genetic variation exhibited by *C. lindemuthianum*, stacking major resistance genes appears to be the best option for developing cultivars with durable anthracnose resistance.

Introduction

Anthracnose is one of the most important diseases of common bean (*Phaseolus vulgaris*) in subtropical and temperate regions, especially under cool and humid conditions (Kelly et al., 1994; Pastor-Corrales, 1988). The disease is endemic in Africa, Australia, Asia and many countries in Latin America (Araya, 1989; Pastor-Corrales, 1988). When infection occurs early in the growth cycle of susceptible cultivars, yield loss can be 100% (Araya, 1989; Fernández et al., 2000; Pastor-Corrales, 1988; Shao and Teri, 1985). The causal agent of anthracnose, *Colletotrichum lindemuthianum*, is an imperfect fungus that is highly variable pathogenically (Araya et al., 1989; Balardin et al., 1997, 1999; Melotto et al., 2000; Menezes and Dianese, 1988; Pastor-Corrales et al., 1993; Sharma et al., 1999). The high variability of *C. lindemuthianum* has resulted in continuous breakdown of resistance in commercial cultivars (Kelly et al., 1994; Menezes and Dianese, 1988; Pastor-Corrales et al., 1993), has complicated the use of host resistance genes (Melotto et al., 2000) and has made it difficult to develop or design effective anthracnose control strategies.

Because the development of resistant cultivars depends; to a large extend, on levels of pathogenic variability exhibited by the causal fungus (Milgroom and Fry, 1997), studies on the variability of *C. lindemuthianum* are needed to direct breeding efforts towards long-term resistance to anthracnose. Variability in *C. lindemuthianum* has been assessed based on the reaction of the isolate on a standard differential series of 12 common bean genotypes and a binary system based on the position of each cultivar within this series (Pastor-Corrales, 1991). The adoption of this standard procedure allows comparison

of data from different research groups. However, the major limitation is that the experiments depend heavily on environmental conditions and in situations where there is no optimization of experimental conditions, the resulting data might not be satisfactory. The use of molecular techniques for the detection of genetic variability offers a complementary and alternative solution for determining genetic variability in pathogen populations. Combining virulence and molecular analysis will lead to a better understanding of the variability present in *C. lindemuthianum,* information that is crucial for designing anthracnose management strategies, deploying resistance genes and developing resistant commercial type bean cultivars.

To elucidate the genetic diversity of *C. lindemuthianum* from diverse regions of the world where common bean anthracnose is an important disease and determine how the variability within *C. lindemuthianum* populations was structured, genomic fingerprints were generated via the polymerase chain reaction (PCR), using random amplified microsatellites (RAMS) (Hantula et al., 1996; Müller and Hantula, 1998), and primers derived from the DNA sequence of repetitive-elements (Rep)-PCR (Jedryczka et al., 1999; Bruijn, 1992). For Rep-PCR analysis, primers derived from the 'enterobacterial repetitive intergenic consensus' (ERIC) sequence and the conserved repeated bacterial DNA element 'BOX' were used (Bruijn, 1992; Jedryczka et al., 1999). The pathotype variability of the *C. lindemuthianum* isolates used in this study was assessed using differential interaction on a set of 12 bean varieties, comprising four Andean and eight Mesoamerican genotypes.

This paper reports the results obtained from analyzing 200 *C. lindemuthianum* isolates collected from Andean and Mesoamerican varieties and from different regions and attempts to elucidate the pathogen population structure as it relates to common bean gene pools.

Materials and methods

Selection of C. lindemuthianum *isolates*

Two hundred isolates of *C. lindemuthianum* were selected from the collection held at CIAT. Selection was based on the gene pool of the variety from which the isolate was collected (Andean or Mesoamerican) and also to represent the widest geographical distribution represented by regions where the disease is prevalent (Table 1). Lyophilized monosporic cultures were revived by adding a sucrose–peptone solution (10% peptone; 20% sucrose) to make a spore suspension that was plated on potato dextrose agar (PDA) and incubated at ∼20 °C (Pastor-Corrales et al., 1993).

Inoculum production and virulence characterization

The virulence phenotype of each monosporic isolate was confirmed by inoculating the standard set of 12 bean differential varieties (Table 1). Each isolate was cultured on PDA and incubated at 20 °C for 7 days in darkness. To harvest fungal spores, PDA plates were flooded with 5 ml of sterile distilled water, the surface scraped with a sterile glass rod and the spores were filtered through four layers of cheesecloth. The spore concentration was adjusted to 1.2×10^6 conidia ml^{-1} using sterile distilled water.

Ten-days-old bean seedlings, with fully expanded primary leaves, were sprayed with the conidia suspension until runoff on the stem and both surfaces of the unifoliate leaves. Inoculated plants were incubated in a chamber at 22 °C and 90–100% relative humidity. Disease reactions were scored 8 days after inoculation based on a 1–9 CIAT scale (Schoonhoven and Pastor-Corrales, 1987), where plants with no visible symptoms or with only a few, very small lesions mostly on the primary leaf veins were recorded as resistant (scales 1–3). Plants with numerous small or enlarged lesions, or with sunken cankers on both the lower sides of leaves and the seedling stem were recorded as susceptible (scales 6.1–9). Intermediate values (3.1–6) were considered as susceptible for the purpose of this work. There were 10 plants of each differential genotype per experiment. The experiment was repeated when variable results were obtained for an isolate among the 10 inoculated plants. A severity value of each genotype was calculated as the mean score of 10 plants. In this system, the differential genotypes were planted in the same order and assigned a numerical value when the reaction is susceptible (Table 1). The values of the susceptible variety were added to give the pathotype designation of the isolate.

Production of mycelium and DNA extraction

Colletotrichum lindemuthianum mycelium was produced in liquid V8 juice medium (Mahuku et al., 2002). Briefly, Erlenmeyer flasks (200 ml) containing

Table 1. Designation, virulence phenotype and origin of 90 pathotypes of *C. lindemuthianum* identified among 200 isolates analyzed during this study

Race	Anthracnose differential cultivars[1]												Country of origin[2] (number of isolates)
	A	B	C	D	E	F	G	H	I	J	K	L	
0	−	−	−	−	−	−	−	−	−	−	−	−	ARG (1), CRI (1), ECU (5), PER (1), HND (1)
1	+	−	−	−	−	−	−	−	−	−	−	−	ARG (1), BRA (1), COL (1), CRI (1), ECU (3), HND (1), PER (1)
2	−	+	−	−	−	−	−	−	−	−	−	−	ARG (1), PER (1)
3	+	+	−	−	−	−	−	−	−	−	−	−	AFR (1), ARG (2), COL (3), DOM (1), MEX (1), PER (2), USA (1)
4	−	−	+	−	−	−	−	−	−	−	−	−	ARG (1), ECU (4), PER (2)
5	+	−	+	−	−	−	−	−	−	−	−	−	ARG (1), COL (1), ECU (1), MEX (1), PER (1)
6	−	+	+	−	−	−	−	−	−	−	−	−	ARG (1), ECU (2), PER (1)
7	+	+	+	−	−	−	−	−	−	−	−	−	ARG (1), COL (3), CRI (1), ECU (1), HND, NIC (1), PER (1)
9	+	−	−	+	−	−	−	−	−	−	−	−	BRA (1), COL (2), CRI (2), ECU (1), GTM (1), HND (2), MEX (1), PER (1)
11	+	+	−	+	−	−	−	−	−	−	−	−	BRA (1), COL (1)
15	+	+	+	+	−	−	−	−	−	−	−	−	COL (1), ECU (1)
17	+	−	−	−	+	−	−	−	−	−	−	−	BRA (1)
23	+	+	+	−	+	−	−	−	−	−	−	−	BRA (1), EUR (1)
31	+	+	+	+	+	−	−	−	−	−	−	−	BRA (1)
36	−	−	+	−	−	+	−	−	−	−	−	−	ARG (1)
38	−	+	+	−	−	+	−	−	−	−	−	−	CRI (1), DOM (1), ESP (3)
39	+	+	+	−	−	+	−	−	−	−	−	−	DOM (1)
47	+	+	+	+	−	+	−	−	−	−	−	−	DOM (1)
64	−	−	−	−	−	−	+	−	−	−	−	−	ARG (1)
65	+	−	−	−	−	−	+	−	−	−	−	−	ARG (1), BRA (1), ECU (1)
73	+	−	−	+	−	−	+	−	−	−	−	−	CRI (1), GTM (1), MEX (4), PRI (1), USA (1)
81	+	−	−	−	+	−	+	−	−	−	−	−	JAP (1)
87	+	+	+	−	+	−	+	−	−	−	−	−	ARG (1)
89	+	−	−	+	+	−	+	−	−	−	−	−	CRI (1)
121	+	−	−	+	+	+	+	−	−	−	−	−	BRA (1)
128	−	−	−	−	−	−	−	+	−	−	−	−	ECU (1), PER (1)
129	+	−	−	−	−	−	−	+	−	−	−	−	COL (1), CRI (1), ECU (1), HND (1), MEX (1)
132	−	−	+	−	−	−	−	+	−	−	−	−	ARG (1), PER (1)
133	+	−	+	−	−	−	−	+	−	−	−	−	ARG (1), COL (1), ECU (1), PER (1)
137	+	−	−	+	−	−	−	+	−	−	−	−	COL (1), CRI (1), HND (1)
139	+	+	−	+	−	−	−	+	−	−	−	−	COL (1)
192	−	−	−	−	−	−	+	+	−	−	−	−	MEX (1)
256	−	−	−	−	−	−	−	−	+	−	−	−	ECU (2), MEX (2)
257	+	−	−	−	−	−	−	−	+	−	−	−	MEX (1)
261	+	−	+	−	−	−	−	−	+	−	−	−	ECU (2)
320	−	−	−	−	−	−	+	−	+	−	−	−	MEX (1)
385	+	−	−	−	−	−	−	+	+	−	−	−	COL (2), MEX (1)
388	−	−	+	−	−	−	−	+	+	−	−	−	COL (1)
393	+	−	−	+	−	−	−	+	+	−	−	−	CRI (1), MEX (1)
448	−	−	−	−	−	−	+	+	+	−	−	−	MEX (1)
449	+	−	−	−	−	−	+	+	+	−	−	−	MEX (1)
453	+	−	+	−	−	−	+	+	+	−	−	−	MEX (1)
457	+	−	−	+	−	−	+	+	+	−	−	−	CRI (1)
513	+	−	−	−	−	−	−	−	−	+	−	−	BRA (1)
515	+	+	−	−	−	−	−	−	−	+	−	−	COL (2)
517	+	−	+	−	−	−	−	−	−	+	−	−	COL (1)
521	+	−	−	+	−	−	−	−	−	+	−	−	COL (3), CRI(1)
523	+	+	−	+	−	−	−	−	−	+	−	−	HND (1)

Table 1. (*Continued*)

Race	Anthracnose differential cultivars[1]												Country of origin[2] (number of isolates)
	A	B	C	D	E	F	G	H	I	J	K	L	
525	+	−	+	+	−	−	−	−	−	+	−	−	COL (1)
529	+	−	−	−	+	−	−	−	−	+	−	−	BRA (1)
535	+	+	+	−	+	−	−	−	−	+	−	−	BOL (1), BRA (1)
593	+	−	−	−	+	−	+	−	−	+	−	−	AFR (1)
641	+	−	−	−	−	−	−	+	−	+	−	−	COL (1)
647	+	+	+	−	−	−	−	+	−	+	−	−	COL (1)
651	+	+	−	+	−	−	−	+	−	+	−	−	COL (2)
653	+	−	+	+	−	−	−	+	−	+	−	−	COL (1)
905	+	−	−	+	−	−	−	+	+	+	−	−	CRI (1)
1025	+	−	−	−	−	−	−	−	−	−	+	−	CRI (1), GTM (1)
1033	+	−	−	+	−	−	−	−	−	−	+	−	COL (1), CRI (1)
1049	+	−	−	+	+	−	−	−	−	−	+	−	CRI (1)
1088	−	−	−	−	−	−	+	−	−	−	+	−	MEX (1)
1089	+	−	−	−	−	−	+	−	−	−	+	−	MEX (1)
1093	+	−	+	−	−	−	+	−	−	−	+	−	MEX (1)
1097	+	−	−	+	−	−	+	−	−	−	+	−	MEX (1)
1153	+	−	−	−	−	−	−	+	−	−	+	−	CRI (1), ECU (1)
1161	+	−	−	+	−	−	−	+	−	−	+	−	CRI (1)
1217	+	−	−	−	−	−	+	+	−	−	+	−	HND (1)
1417	+	−	−	+	−	−	−	+	+	−	+	−	HND (1)
1433	+	−	−	+	+	−	−	+	+	−	+	−	CRI (1)
1435	+	+	−	+	+	−	−	+	+	−	+	−	CRI (1)
1473	+	−	−	−	−	−	+	+	+	−	+	−	HND (1), MEX (1)
1481	+	−	−	+	−	−	+	+	+	−	+	−	CRI (1)
1489	+	−	−	−	+	−	+	+	+	−	+	−	CRI (1)
1497	+	−	−	+	+	−	+	+	+	−	+	−	CRI (1)
1545	+	−	−	+	−	−	−	−	−	+	+	−	COL (3), CRI (1), GTM (1)
1549	+	−	+	+	−	−	−	−	−	+	+	−	GTM (1)
1561	+	−	−	+	+	−	−	−	−	+	+	−	CRI (1)
1609	+	−	−	+	−	−	+	−	−	+	+	−	CRI (1)
1645	+	−	+	+	−	+	+	−	−	+	+	−	GTM (1)
1929	+	−	−	+	−	−	−	+	+	+	+	−	CRI (2)
1945	+	−	−	+	+	−	−	+	+	+	+	−	CRI (1)
1985	+	−	−	−	−	−	+	+	+	+	+	−	CRI (1)
1993	+	−	−	+	−	−	+	+	+	+	+	−	CRI (1)
2001	+	−	−	−	+	−	+	+	+	+	+	−	CRI (1)
2009	+	−	−	+	+	−	+	+	+	+	+	−	CRI (1)
2047	+	+	+	+	+	+	+	+	+	+	+	−	CRI (1)
3481	+	−	−	+	+	−	−	+	+	−	+	+	CRI (4), ARG (1)
3545	+	−	−	+	+	−	+	+	+	−	+	+	CRI (1)
3977	+	−	−	+	−	−	−	+	+	+	+	+	CRI (1)
3993	+	−	−	+	+	−	−	+	+	+	+	+	ARG (1)

[1]Common bean differential genotypes used to designate *C. lindemuthianum* pathotypes followed by their binary value and resistance genes they carry in brackets (Goncales-Vidigal et al., 2003; Melotto et al., 2002; Pastor-Corrales, 1991): A: Michelite (1; ?); B: Michigan Dark Red Kidney (2; *Co-1*); C: Perry Marrow (4; *Co-1*^{3}); D: Cornell 49242 (8; *Co-2*); E: Widusa (16; *Co-1*^{5}); F: Kaboon (32; *Co-1*^{2}); G: Mexico 222 (64; *Co-3*); H: PI 207262 (128; *Co-4*^{3}; *Co-9*); I: TO (256; *Co-4*); J: TU (512; *Co-5*); K: AB 136 (1024; *Co-6, co-8*) and L:G2333 (2047; *Co-4*^{2}, *Co-5, Co-7*). Andean genotypes are Michigan Dark Red Kidney, Perry Marrow, Widusa and Kaboon; + = susceptible; − = resistant.

[2]Country of origin: ARG = Argentina, PER = Peru, ECU = Ecuador, BRA = Brazil, COL = Colombia; CRI = Costa Rica, NIC = Nicaragua, GTM = Guatemala, HND = Honduras, MEX = Mexico, PRI = Puerto Rico, DOM = Dominican Republic, USA = United States of America, EUR = Europe, ESP = Spain, JAP = Japan and AFR = Africa. The number in parenthesis is the total number of isolates from that country in the designated pathotype.

60 ml of liquid V8 juice medium were inoculated with 10 agar disks of 2 mm in diameter and cut from edges of actively growing fungi on PDA. The cultures were placed on a rotary shaker (160 rpm) at room temperature (~21 °C) for 8 days. Mycelium was vacuum filtered through two layers of cheesecloth, washed in sterile deionized water, freeze-dried and stored at −80 °C. DNA was extracted from 0.25 to 0.3 g freeze-dried mycelia (Möller et al., 1992, as modified by Mahuku et al., 2002). The DNA was electrophoresed in 0.7% agarose gels to determine the quality, and DNA quantified using a fluorometer (Hoefer® DyNA Quant 2000, Pharmacia Biotech, USA) and adjusted to a standard concentration of 5 ng μl^{-1} in 0.1 × TE buffer.

RAMS analysis

Eight RAMS primers ((CCA)n, 5′-DDB$(CCA)_5$-3′; (GT)n, 5′-VHV$(GT)_5$G-3′; (AG)n, 5′-HBH$(AG)_7$A-3′; (CT)n, 5′-DVD$(CT)_7$C-3′; (TG)n, 5′-HVH$(TG)_7$T-3′; (CA)n, 5′-DBDA$(CA)_7$-3′; (ACA)n, 5′-BDB$(ACA)_5$-3′; (CGA)n, 5′-DHB$(CGA)_5$-3′) that exhibited polymorphisms and yielded consistent scorable banding patterns, were used to amplify DNA. RAMS PCR reactions were carried out in 12.5 µl volumes containing 1× DNA polymerase buffer (50 mM Tris–HCl (pH 8.5), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 0.2 mM of each dNTP, 0.4 µM of primer, 1 U AmpliTaq DNA polymerase (Perkin–Elmer, NJ, USA) and 400 pg of genomic DNA. A water control (DNA replaced with sterile water) was included with each set of 20 isolates. Ten microliter of light mineral oil were layered over the reaction mixture to prevent evaporation of samples. DNA amplification was performed in a programmable Thermal Cycler (MJ Research, Waterhouse, MA) under a program of one cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 58 °C (GT), 61 °C (CGA), 50 °C (AG), 55 °C (TG), 41 °C (CA, and CT), 49 °C (ACA) and 55 °C (CCA) for 1 min and 72 °C for 2 min and a final 10 min extension at 72 °C.

Rep-PCR analysis of C. lindemuthianum *isolates*

Two types of markers (ERIC (ERIC-1R 5′-ATGTA AGCTCCTGGGGATTCAC-3′ and ERIC-2 5′-AAG TAAGTGACTGGGGTGAGCG-3′) and BOX-AIR 5′-CTACGGCAAGGCGACGCTGACG-3′) were used (Jedryezka et al., 1999). The reactions were carried out in 12.5 µl, containing 0.2 mM of each dNTPs, 1 × PCR buffer (Gitschier buffer), 0.5 U of Taq DNA polymerase (Perkin–Elmer, NJ, USA), 0.2 mM each primer, 1.5 mM Mg^{+2}, 0.16 mg ml^{-1} BSA, 10% DMSO, 20 ng genomic DNA and 20 µl of light mineral oil.

Gel electrophoresis

The amplification products were electrophoresed in 1× Tris–borate–EDTA buffer (45 mM Tris–borate, 0.127 mM EDTA pH 8.0) containing ethidium bromide (0.17 µg ml^{-1}) for 6 h at 60 V at room temperature. DNA fragments were separated in 1.2% (w/v) agarose gel containing ethidium bromide (0.24 µg ml^{-1}) and photographed under 300 nm UV light. Gel images were captured using the Eagle Eye II 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). Replicate reactions (different PCR runs) were run on separate gels for each primer–isolate combination. For each primer, non-reproducible fragments between three repetition tests were discarded. Comparison of profiles for each primer was done on the basis of the presence or absence of fragments.

Statistical analysis

Virulence and molecular data were analyzed separately. A data matrix was generated from the virulence phenotypes by considering incompatible interactions (rating ≤ 3) as absence of a virulence marker (0) and compatible interactions (rating >3) as presence of a marker (1) and each differential as a different marker. Similarly, RAMS and Rep-PCR markers were scored as either present (1) or absent (0) of a band and only strong and reproducible bands were scored, faint bands were discarded. The genetic distance between two isolates was calculated based on Dice's coefficient using SAS program (SAS version 6, 1989). Multiple correspondence analyses (MCA) was used to assign isolates to virulence or haplotype clusters, to avoid defining every clonally related pair of haplotyes as a separate group. Each MCA defined group of isolates was considered as a separate genetic lineage. Correlation between virulence and RAPD lineages were determined using the MXCOMP option of NTSYS and Spearman's rank correlation coefficients.

Genetic diversity

To calculate the genetic diversity, isolates were divided into groups based on cluster and MCA. Genetic diversity was calculated using Equation (1) (Leung et al., 1993):

$$H = 1 - \sum f(i)^2 \quad (1)$$

where $f(i)$ represents the frequency of band i in the population and $\sum f(i)^2$ is the probability that two individuals taken from the population at random, have band i. The mean of these values, defined as H_S estimates the diversity that exists within subpopulations or subgroups and was calculated using Equation (2):

$$H_S = \frac{(n_1 H_1 + n_2 H_2 + \cdots + n_j H_j)}{(n_1 + n_2 + \cdots + n_j)} \quad (2)$$

where n_j represents the number of individuals in group j, and H_j is the genetic diversity in subgroup H_j. The coefficient of genetic diversity (G_{ST}) estimates the proportion of total variability observed between groups and was estimated as:

$$G_{ST} = \frac{(H_T - H_S)}{H_T} \quad (3)$$

where H_T represents total diversity and H_S represents the mean diversity within subgroups or subpopulations. In addition, analysis of molecular variance (AMOVA) was used to partition the total genetic variation between Andean and Mesoamerican groups of *C. lindemuthianum* assigned based on the host genotype the isolates were collected from and the virulence phenotype.

Results

Virulence analysis

Ninety pathotypes (races) were described among the 200 *C. lindemuthianum* isolates that were studied (Table 1). Most of the pathotypes (54) were restricted to a single country, whereas, 36 were found in at least two countries. Pathotype 9 was the most frequent and widely distributed, being recovered from both Andean and Mesoamerican genotypes and from eight countries (Brazil, Colombia, Costa Rica, Ecuador, Guatemala, Honduras, Mexico and Peru) (Table 1). Other pathotypes that had high frequencies were 0, 3 and 7, with a recovery frequency of 4.5% each (Table 1). Most pathotype diversity (40%) was found in Costa Rica, followed by Colombia with 25.5%.

Following the hypothesis that Andean isolates are only able to infect Andean genotypes, 22 isolates that were distributed into five pathotypes (2, 4, 6, 36 and 38) were identified as Andean while the rest 84 (172 isolates) were classified as Mesoamerican (Tables 1 and 2). Nine isolates were identified as pathotype zero (0) and had no compatible interaction with any of the differential genotypes, except the universal susceptible variety La Victoire (Tables 1 and 2). These isolates were excluded from the host pathogen co-evolution analysis because they did not fit into any of the two groups.

A weak correlation ($r = 0.11$; $P = 0.311$) was observed between group designation of the pathotype and the gene pool of the host from where the isolate was collected (Table 2). None of the isolates collected from Mesoamerican genotypes were classified as Andean while 42 isolates (32 pathotypes) collected from Andean varieties were classified as Mesoamerican (Tables 1 and 2).

Multiple correspondence analysis of virulence data

Multiple correspondence analysis separated isolates into four groups that were not structured with host gene pool or with geographical origin (Figure 1). For example, group 1 contained 157 isolates, of which 49 had been collected from Andean genotypes and 106 from Mesoamerican genotypes and from different geographical regions. The same trend was true for groups 2 and 3, while group 4 contained a single isolate representing pathotype 2047. Analysis of pathotype diversity showed that 96.6% of the pathotype diversity was within groups defined by MCA, compared to 3.4% between groups. There was apparently no genetic differentiation ($G_{ST} = 0.034$) between virulence groups of *C. lindemuthianum*, showing that virulence analysis did not separate isolates into groups congruent with bean gene pools. Based on their virulence phenotype, isolates from Central America were very diverse (Table 1). Assuming that each differential genotype contains a different resistance gene(s) or allele(s), all virulence genes found in the Andean population were also present in the Mesoamerican population, while some virulence genes were present only in the Mesoamerican region (Table 1).

Table 2. Classification of 200 *C. lindemuthianum* isolates according to common bean gene pools, Andean and Mesoamerican

Country of origin	Number of isolates	Host gene pool of isolate collection[1]	Phenotype[2]
Africa	2	A	M
	4	A	A
Argentina	11	A	M
	1	A	—
Bolivia	1	M	M
Brazil	11	M	M
Colombia	11	A	M
	24	M	M
Costa Rica	1	M	—
	1	A	M
	39	M	M
Dominican Republic	3	A	M
	1	A	A
Ecuador	5	A	—
	10	A	A
	9	A	M
	3	M	M
Spain	3	A	A
Europe	1	A	M
Guatemala	6	M	M
Honduras	1	M	—
	10	M	M
Japan	1	M	M
Mexico	23	M	M
Nicaragua	1	M	M
Peru	1	A	—
	4	A	A
	9	A	M
Puerto Rico	1	M	M
USA	2	M	M

[1]Host gene pool of isolate collection; A = Andean, M = Mesoamerican.

[2]Phenotype is the pathotype classification following isolate characterization on a set of 12 standard anthracnose differential genotypes (Pastor-Corrales, 1991); A = Andean, M = Mesoamerican, — = isolates classified as pathotype zero, did not infect any of the differential genotypes except the universal susceptible variety, La Victoire.

RAMS analysis

The eight RAMS primers generated simple banding patterns that showed a high level of polymorphisms between the 200 *C. lindemuthianum* isolates. Figure 2 gives an example of the amplification profiles observed

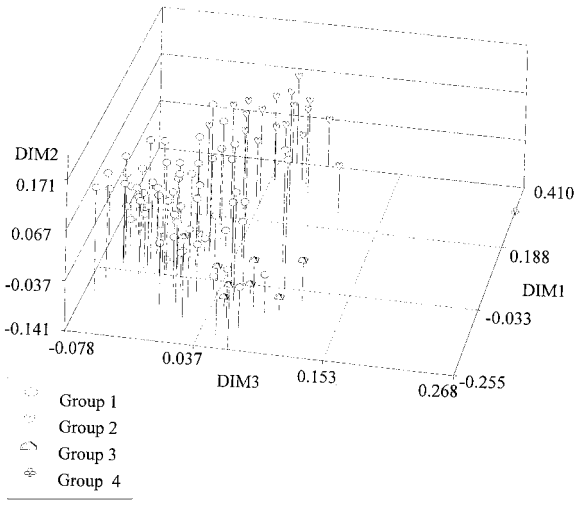

Figure 1. Three-dimensional graph based on MCA of virulence characterization on a set of 12 bean differential varieties and plotted using the spin platform of JMP program in SAS. Symbols indicate position of pathotypes within each cluster.

when using the RAMS primer (TG)n. A total of 206 bands were scored, of which, 142 (60%) were polymorphic. Bands that had a frequency more than 1% and less than 99% were considered as polymorphic. Cluster analysis identified 199 haplotyes that were distributed into four groups following MCA (Figure 3A). Isolates belonging to the same pathotype often had different molecular patterns and at times, were distributed into different groups. Most of the isolates (98%) were found in clusters 1 and 2, whereas clusters 3 and 4 had two and one isolates, respectively. Of the 93 isolates in group 1, only one isolate was classified as Andean whereas 21 of the 104 isolates in group 2 were Andean. Thus, the *C. lindemuthianum* isolates could not be separated into Andean and Mesoamerican groups. There was no correlation between groups that were defined following analysis of virulence and RAMS data ($r = 0.05$; $P < 0.05$).

Analysis of Rep-PCR

Rep-PCR generated products of 0.2–2.5 kb. Figure 4 shows an example of the amplification using BOX-AIR primers. A total of 174 haplotypes, which were distributed into four clusters were defined for the 200 *C. lindemuthianum* isolates. Most of the haplotypes (99.5%) were distributed in groups 1, 2 and 3, while group 4 consisted of a single haplotype (Figure 3B). There was no apparent separation or clustering of

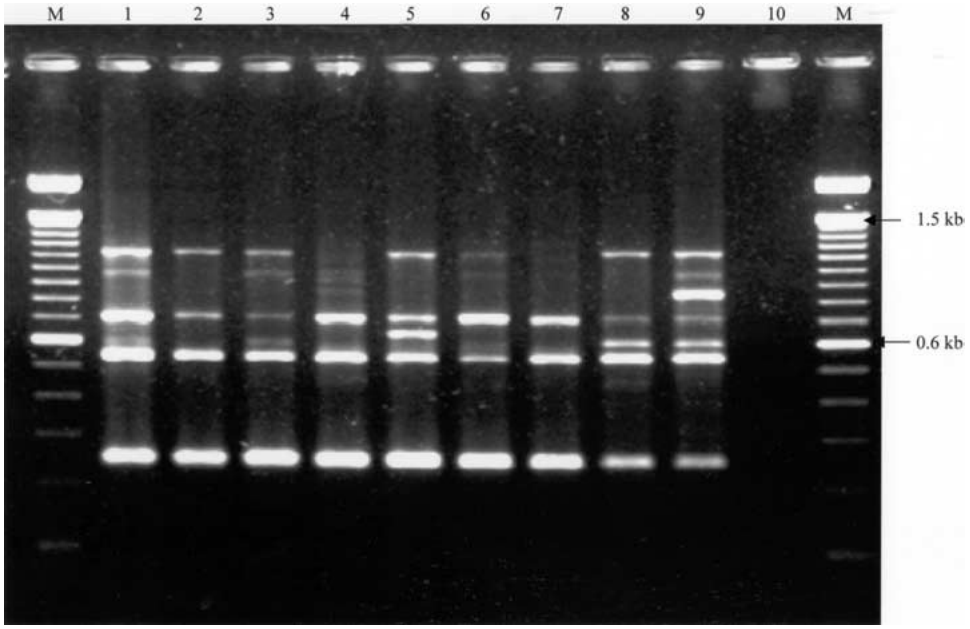

Figure 2. Random amplified microsatellite profiles of *C. lindemuthianum* isolates generated using primer (CA)n. Lanes 1–4 represents *C. lindemuthianum* Andean isolates while lanes 5–9 represent Mesoamerican isolates. Lane 10 is negative control while lane M represents the 100 bp DNA molecular size marker.

the isolates according to the geographical origin, their host gene pool or their virulence phenotype. No correlations between groups defined by virulence analysis and those resulting from Rep-PCR were apparent ($r = 0.03$; $P < 0.05$). However, Rep-PCR and RAMS were highly correlated ($r = 0.52$; $P = 0.01$).

Analysis of genetic diversity

Genetic diversity analysis using molecular data showed that *C. lindemuthianum* is a highly variable pathogen ($HT = 0.993$). Most of the observed genetic variation (0.98) was attributed to differences between isolates in the same group, compared to (0.02) that resulted from differences between isolates in different groups. AMOVA following partitioning of isolates according to the host gene pool from which they were collected showed that most of the genetic variation (89%) was among isolates within a group compared to 11% between groups. However, when isolates were separated into groups after virulence characterization, 91.5% of the genetic variation was ascribed to variation among isolates within groups compared to 8.5% between them. No significant genetic differentiation ($G_{ST} = 0.02$) was observed between *C. lindemuthianum* groups.

Discussion

Molecular and virulence markers were used to elucidate the level of genetic diversity existing in *C. lindemuthianum* populations collected from diverse regions of the world. Results reveal that *C. lindemuthianum* is a highly variable pathogen that maintains high levels of genetic diversity. While some studies (Balardin and Kelly, 1998; Pastor-Corrales, 1996; Sicard et al., 1997) reported specialization of *C. lindemuthianum* on the major gene pools of *P. vulgaris*, our results showed that *C. lindemuthianum* isolates do not separate into groups that are congruent with host gene pools defined for common bean. Furthermore, no geographical differentiation was evident for *C. lindemuthianum*. These results support the conclusions by Balardin et al. (1997; 1999) and Fabre et al. (1995) who reported a lack of geographical differentiation and could not conclusively establish that *C. lindemuthianum* co-evolved with gene pools established for *P. vulgaris*.

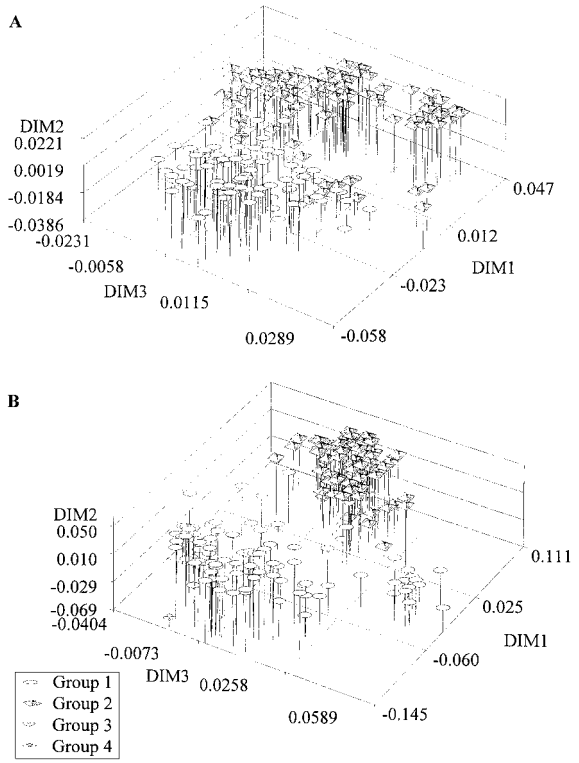

Figure 3. Three-dimensional graph based on MCA of (A) RAMS and (B) Rep-PCR data and plotted using the spin platform of JMP program in SAS. Symbols indicate position of strains within each cluster.

Although some pathotypes were only recovered in regions that cultivate Andean beans (9 pathotypes) or Mesoamerican beans (52 pathotypes), a significant number of pathotypes (29) were common to both regions. This is not surprising as Mesoamerican isolates have been demonstrated to have a wider virulence spectrum than Andean isolates. However, Mesoamerican isolates were isolated from Andean genotypes in Andean regions (Peru, Ecuador and Colombia). It has been hypothesized that *C. lindemuthianum* originated and was disseminated from the Mesoamerican region. This hypothesis is supported by the work done by Sicard et al. (1997), who when using RAPDs and RFLP of the ITS region found that all *C. lindemuthianum* alleles in an Ecuadorian and Argentinean population were also found in a Mexican population. However, more alleles were found only in the Mexican population. They concluded that the Ecuadorian and Argentinean populations probably arose from the Mexican population through founder effects and genetic drift. The results reported in this study support this hypothesis and we can therefore, hypothesize that the ancestral population of *C. lindemuthianum* is found in the Mesoamerican region.

Based on the interaction of 62 bean genotypes and 34 *C. lindemuthianum* pathotypes, Balardin and Kelly (1998) concluded that selection of virulence factors were congruent with the diversity in *P. vulgaris*.

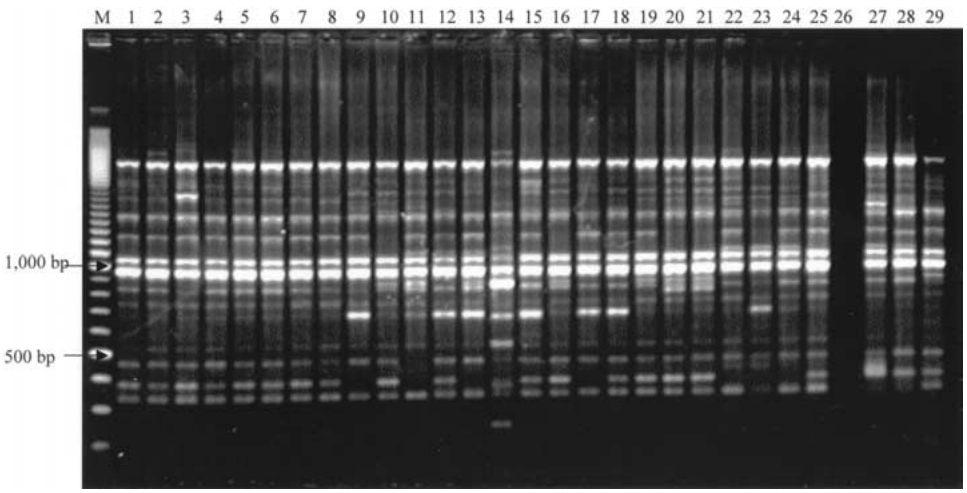

Figure 4. Banding patterns of some *C. lindemuthianum* isolates collected from Andean and Mesoamerican common bean genotypes and generated by ERIC-primers. Lanes 26 is negative control (no *C. lindemuthianum* DNA added) and lane M is the 100 bp DNA molecular size marker.

However, most of the bean genotypes used in this study were bred or improved germplasm. It has been reported that the response of a genotype to infection is dependent on the origin of the resistance gene carried rather than the gene pool to which the variety belongs (Balardin and Kelly, 1998). Therefore, the use of bred genotypes, where the identity and origin of the resistance genes are not known might confound the classification of isolates into distinct groups congruent with the diversity in *P. vulgaris*. Our study does not reveal specialization of *C. lindemuthianum* on a particular bean gene pool. However, it is important to clarify that the separation and classification of pathotypes is dependent on the differential varieties used (Leung et al., 1993). The present *C. lindemuthianum* differential series contains only four Andean genotypes, all carrying the *Co-1* resistance gene or its alleles. A thorough and detailed analysis based on a larger set of Andean and Mesoamerican wild bean genotypes, is warranted before strong conclusions about co-evolution of *C. lindemuthianum* and common bean gene pools can be made.

Colletotrichum lindemuthianum displayed a high level of pathotype diversity and this diversity was greatest from regions cultivating Mesoamerican beans. Similar observations have been reported in other studies (Araya, 1989; Menezes and Dainese, 1988; Pastor-Corrales et al., 1993; Sicard et al., 1997), demonstrating that *C. lindemuthianum* possesses the greatest variability in the Mesoamerican region. The level of pathotype diversity displayed is either a reflection of the dynamic nature of preexisting pathotypes or it reflects the continued evolution of pathotypes in response to the introduction of new resistance genes (directional selection) (Fabre et al., 1995; McDonald and Linde, 2002).

The high levels of genetic diversity observed for *C. lindemuthianum* are common for sexually reproducing pathogens, where recombination is an important factor in generating new genotypes. Even though the sexual form of *C. lindemuthianum* has been described under laboratory conditions (Kimati and Galli, 1970), it has never been detected for natural populations. In this case, sexual reproduction might be infrequent or absent in nature, making *C. lindemuthianum* exclusively asexual. *C. lindemuthianum* is seed-borne (Pastor-Corrales et al., 1993) and movement of new pathotypes occur through contaminated seed. This, coupled with selection over time, and driven by the introduction of new varieties with new sources of resistance (Pastor-Corrales et al., 1993) will result in higher levels of pathotype diversity. Gene flow, mitotic and/or parasexual reproduction among other factors (Leung et al., 1993), could be playing major roles in generating and maintaining the high genetic variation observed in this pathogen.

While confirming the high genetic diversity maintained by *C. lindemuthianum,* our study provides evidence that *C. lindemuthianum* is not a pathogen that is specialized on a particular *P. vulgaris* gene pool. Furthermore, the genetic structure of *C. lindemuthianum* reveals no geographical differentiation. This has important implications in deployment of resistance genes and directing programs tasked with developing anthracnose resistant cultivars. Even though geographical specialization was not evident, the greatest variability of the pathogen was observed from regions or areas that normally or typically cultivate Mesoamerican beans. As such, the greatest challenge to manage anthracnose of common bean is in the Mesoamerican region and it is here that pyramiding or stacking resistance genes with greatest effect against *C. lindemuthianum* will have the greatest impact.

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