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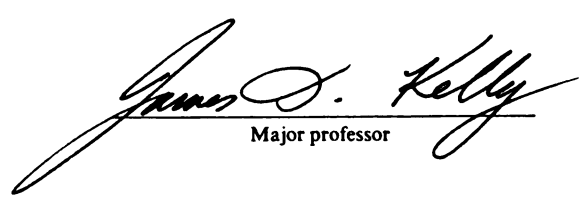

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TRADITIONAL AND MOLECULAR CHARACTERIZATION OF VARIABILITY IN *COLLETOTRICHUM LINDEMUTHIANUM*

By

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ABSTRACT

TRADITIONAL AND MOLECULAR CHARACTERIZATION OF VARIABILITY IN *COLLETOTRICHUM LINDEMUTHIANUM*

By

Ricardo Silveiro Balardin

Colletotrichum lindemuthianum isolates were characterized into 41 races based on virulence to twelve differential cultivars of *Phaseolus vulgaris*. Races 7, 65, and 73 were widespread. No race was isolated from the Andean and the Middle American gene pools of *P. vulgaris* although 39% of the races were detected multiple times. Phenetic analyses showed no obvious geographical patterns correlated with virulence clusters. Genetic diversity of *C. lindemuthianum* was shown to be the greatest in Central America. Diversity was estimated using sequence homology and RFLP analysis of the ribosomal subunit spacer (ITS), and RAPD analysis of total genomic DNA. Polymorphism in the rDNA spacer region was not linked to any specific genetic factor. Parsimony and neighbor-joining analyses supported a monophyletic group formed by all except race 31. RFLP-ITS analysis placed Andean races predominantly into group I except race 23, which was placed within group II. The Middle American races were observed in both groups. Molecular polymorphism among isolates of similar virulence phenotype revealed a level of molecular variability within *C. lindemuthianum* greater than the variability characterized using virulence analysis. Thirty-four races of *C. lindemuthianum* were

inoculated on sixty-two cultivars of *P. vulgaris*. Bean genotypes clustered based on the gene pool origin of the resistance genes present, regardless of the actual gene pool of the host genotype. Races of *C. lindemuthianum* with Middle American reaction showed broad virulence on germplasm from both gene pools, whereas races with Andean reaction showed high virulence only on Andean germplasm. The reduced virulence of Andean races on Middle American genotypes suggests selection of virulence factors congruent with diversity in *P. vulgaris*. The majority of races of *C. lindemuthianum* grouped according to specific gene pool (i.e. Middle American and Andean) based on principal component analysis, except a small group of isolates which appeared to possess factors of virulence to both host gene pools. No apparent geographic effect was observed. Virulence data supported variability in *C. lindemuthianum* structured with diversity in *Phaseolus*, whereas molecular data showed no congruence between pathogen and host populations.

DEDICATION

To my parents, my inspiration.

To my wife, Clarice, my strength and support all time long.

To my children, Ricardo and Gabriela, I hope they have dignity and force to get the best in their lives.

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My family, for the unconditional support and love.

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GENERAL INTRODUCTION

Anthracnose is caused by the anamorphic fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams. -Scrib. The teleomorphic phase, *Glomerella lindemuthiana* (Shear), has not been reported in field conditions. It is an important disease in common bean worldwide responsible for yield losses as high as 95% (Guzman et al., 1979). Infected seeds serve as a primary source of inoculum and optimum conditions for disease development include: early infection on susceptible cultivars associated with high humidity and frequent precipitation (Zaumeyer and Thomas, 1957; Guzman et al., 1979). Anthracnose is an important disease in sub-tropical and temperate regions due to efficient seed transmission (Tu, 1992), and the lack of cost-effective chemical control methods (Pastor-Corrales and Tu, 1989). In addition, the ability of *C. lindemuthianum* to survive in plant debris up to 22 months (Dillard and Cobb, 1993), and the occasional development of sclerotia (Sutton, 1992), limits the effective control of this disease.

Anthracnose control is best achieved through an integrated pest management approach (IPM) based on exclusion and eradication measures (Pastor-Corrales and Tu, 1989; Tu, 1988; Zaumeyer and Thomas, 1959). Exclusion of *C. lindemuthianum* can be accomplished by quarantine, establishment of tolerance levels for infected seeds, and selection of disease free seed production areas. Eradication of *C. lindemuthianum* can be achieved by seed treatment, cultural measures, sanitation procedures, fungicides spraying, and resistant cultivars. In many countries the use of IPM for anthracnose

control is difficult to implement. Traditionally, host resistance has been the most appropriate control for anthracnose. However, the high variability in *C. lindemuthianum* (Pastor-Corrales and Tu, 1989) has resulted in continuous breakdown of resistance in commercial germplasm. Unfortunately, extensive information about variability in *C. lindemuthianum* has been collected at the local level with no standardized procedure. The recommendation to use an international differential series and a unique race designation system was made at the First Workshop on Anthracnose of Common Bean in Latin America (Pastor-Corrales, 1988). Since then, a standardized method has been used to characterize the variability in *C. lindemuthianum*.

Traditional characterization. Barrus (1911, 1918) first described variability in *C. lindemuthianum*. Races alpha and beta were identified based on reaction of 139 bean cultivars. Isolates from different geographic origins showed different virulence phenotypes. Resistant and susceptible cultivars accounted for 6% and 70% of all germplasm, respectively. Burkholder (1923) identified race gamma from one isolate virulent to the cultivar White Imperial, which was reported previously as resistant by Barrus (1918). Andrus and Wade (1942) identified race delta from isolates collected in North Carolina. In Chile, Mujica (1952) reported races alpha, beta and gamma. Blondet (1963), Bannerot (1965), and Charrier and Bannerot (1970) reported presence of races alpha, beta, gamma, delta, epsilon and lambda, in France. Leakey and Simbwa-Bunnya (1972) reported the same group of races in Uganda. Fouilloux (1975), Schnock et al. (1979), and Hubbeling (1977) described races alpha-Brazil, iota, and ebnet virulent to the *Co-2* gene. Later, the race ebnet was designated as race kappa (Kruger et al., 1977).

Hubbeling (1976) reported the race alpha-mutant, later designated lambda. Fouilloux (1979) identified the race lambda-mutant. Tu et al. (1984) and Tu (1988, 1994) reported races epsilon, delta, lambda, and alpha-Brazil in Canada. In Brazil, races alpha, Mexique II, delta (Kimati, 1966), alpha, delta, epsilon, lambda, teta, eta, mu, zeta (Menezes and Dianese, 1988), and alpha-Brazil (Balardin et al., 1990) were identified.

Many research groups have used local cultivars and different codes to describe the variability in *C. lindemuthianum*. In Germany, Peurer (1931) identified A-E, G-N, and X races, which were later reported by Schreiber (1932) as equivalent to races alpha, beta and gamma, respectively. In Australia, Waterhourse (1955) and Cruikshank (1966) identified races 1 to 8, and 1 to 3. No equivalence of the Australian races to any known race has been reported. In Mexico, Yerkes and Teliz-Ortiz (1956) identified groups I to III. Yerkes (1958) reported races MA-11 to MA-13 equivalent to the alpha race. Noyola et al. (1984) designated races MA-21 and MA-22 equivalent to the alpha race. Garrido (1986) related races MA-20 to MA-25 with alpha, and MA-26 to MA-30 with Mexico I. In France, Bannerot (1965) described races PV6, D10, F86, I4, 1 and 5, which might correspond to alpha, beta, gamma, delta, epsilon, and a combination of gamma and delta races, respectively. In Brazil, Oliari et al. (1973) characterized groups Brazilian I and II. Pio-Ribeiro and Chaves (1975) reported races BA-1 to BA-10, and suggested equivalence to races alpha, delta, Mexico I, and Mexico II, and groups Brazilian I and Brazilian II. Schwartz et al. (1982) reported race C-236 identified from an isolate collected in Guatemala. Despite tentative equivalence to known races, data collected using local hosts limited the knowledge of variability in *C. lindemuthianum*.

Standardized methodologies for race identification are recognized as

indispensable to a broader understanding of structure in the *C. lindemuthianum* population. Utilization of an international differential series was suggested (Pastor-Corrales, 1988, 1991; Drijfhout and Davis, 1989). The differential series proposed by Pastor-Corrales (1991) has revealed a wide variability in *C. lindemuthianum*. In the United States, races 7, 64, 65 and 73 were identified (Kelly et al., 1994; Balardin and Kelly, 1996). In Mexico, Garrido (1986) and Rodriguez-Guerra (1991) reported 32 races. In Nicaragua, Rava et al. (1993) identified 9 races from 10 isolates. In Colombia, 33 races were characterized from a group of 178 isolates (Restrepo, 1994).

Since Barrus (1911), breeding programs have used *C. lindemuthianum* virulence phenotypes to screen resistant individuals. However, information related to population structure of this pathogen has been omitted. Because only a few genes are involved in the *C. lindemuthianum* - *P. vulgaris* interaction, limited information is obtained from virulence analyses. In addition, virulence is under selection and may respond to environment changes. Constant monitoring of pathogen variability is needed. Durability of resistance depends on how efficiently new predominant virulence phenotypes are detected within a population, and how fast resistance against the newest race is incorporated into commercial germplasm. Therefore, collecting more extensive information about structure of variability within *C. lindemuthianum* is necessary. Analysis of population structure for making evolutionary inferences using selectively neutral genetic markers is preferred. There is often strong selection for virulence depending on the environment (Milgroom, 1995). Neutral molecular markers can provide information on evolutionary processes such as gene flow, drift or recombination. In contrast, population structure inferred from selectable markers might reflect the selective

pressures operating on a population (Milgroom, 1995; McDonald and McDermott, 1993). Combining virulence and molecular analyses should lead breeding programs towards developing durable resistance to anthracnose in common bean.

Molecular characterization. Molecular genetic markers are selectively neutral, highly informative, reproducible, and relatively easy to assay (McDonald, 1997). Markers based on restriction fragment length polymorphisms (RFLP-based), polymerase chain reaction (PCR-based), and isozymes are the most common molecular markers used in fungal systematics and in population genetics (Michelmore and Hulbert, 1987; Bruns et al., 1991; Milgroom, 1995; Hillis et al., 1996). Mapping and sequencing the ribosomal internal transcribed DNA spacer regions (rDNA-ITS) and the rRNA genes (18S, 5.8S, 28S) have been used in analyses of gene evolution, and studies on inter- and intraspecific phylogenetic relationships, and population structure (Bruns et al., 1991; Hillis et al., 1996).

Molecular analysis of entire genomes can reveal the extent of variability in one species. RFLPs have been used to determine origin and evolution of individual isolates or races within asexual populations exhibiting low levels of recombination (Michelmore and Hulbert, 1987). RFLPs combined with Southern hybridization are more reproducible and more difficult to conduct than RAPDs. They are codominant and exhibit a potentially unlimited number of alleles per locus, what makes RFLP-based markers advantageous compared to RAPDs (McDonald, 1997). Large amounts of DNA from each individual and laborious protocols (cloning, southern blotting, radioactive labeling of probes) are disadvantages of RFLPs. RAPD-PCR technology is an automated method, easy to

implement, and can be used to characterize highly variable pathogens, provided a large bank of random primers are available (Palumbi, 1996).

a. RAPDs. Several advantages of RAPDs have been reported. The technique is fast and simple, independent of gene expression, and able to be transferred between laboratories (Williams et al., 1990, Palumbi, 1996). Another advantage of RAPDs is the large number of different products randomly amplified in the genome. A section of every chromosome generating markers at many loci might be amplified (Palumbi, 1996; Williams et al., 1990). RAPDs have been used in systematics for differentiation and grouping of isolates from different fungal species (Crowhurst et al., 1991; Aufauvre-Brown et al., 1992; Kolmer et al., 1995). Geographic origin and differential virulence in fungi has been corelated to molecular diversity for many species (Megnegneau et al., 1993; Goodwin and Annis, 1991; Nicholson and Rezanoor, 1994; Kolmer et al., 1995). Kolmer et al. (1995) observed a high degree of molecular polymorphism among isolates that had the same virulence phenotype in *Puccinia recondita* f.sp. *tritici*. The molecular polymorphism within virulence phenotypes was considered to be a factor that explained the low correlation between virulence and molecular data. Guthrie et al. (1992) used RAPDs to identify virulence phenotypes of *Colletotrichum graminicola*, and suggested the influence of geographical origin on the variability of this pathogen. Mills et al. (1992) differentiated and grouped isolates of *C. gloeosporioides* based on RAPDs. The effect of host and geographical origin on pathogen variability was demonstrated.

Variability in *C. lindemuthianum* has been characterized by RAPDs. Fabre et al. (1995) grouped *C. lindemuthianum* using RAPDs, and RFLP of PCR-amplified internal

transcribed spacers (ITS) of rDNA. No correlation between DNA polymorphism and the geographic origin of isolates was observed. Using a similar methodology, Sicard et al. (1997) collected isolates of *C. lindemuthianum* from wild common bean populations in Argentina, Ecuador and Mexico. Isolates were divided correspondingly to the *P. vulgaris* gene pools observed in each of these centers of diversity. Restrepo (1994) identified and grouped isolates collected in the Andean and Northern regions of Colombia. Vilarinhos et al. (1996) characterized isolates from Brazil and suggested the use of RAPDs for fast monitoring of *C. lindemuthianum* variability.

Recent reports have pointed out a number of problems associated with assessing phylogenetic relationships using RAPD markers. RAPDs have several technical limitations that make them difficult to reproduce (McDonald, 1997). Some of these limitations can be overcome with replicate DNA preparations, southern analysis, and conversion of RAPD amplicons into sequence characterized amplified regions –SCARS (McDonald, 1997). Conditions of amplification are critical for the interpretation of results, and absence of a product in a particular reaction could be caused by many genomic differences (Palumbi, 1996). Homologous loci are very difficult to identify limiting the use of RAPDs in interpopulational comparisons (Smith et al., 1995). In addition, RAPDs have only two alleles (amplification or nonamplification) at each amplicon locus. Although this is ideal for genetic mapping, it is a limitation for measures of genetic diversity affected by the number of alleles at a locus (McDonald, 1997). Nucleic acid sequencing has been a powerful approach for analysing intraspecific diversity. Sequencing studies of the rRNA genes have proven useful in analysing evolutionary divergences (Hillis et al., 1996). The variable regions in the rRNA genes

make them useful for examining relationships within more closely related groups (Mitchell et al., 1995; Palumbi, 1996).

b. Internal Transcribed Spacers (ITS). Most fungal phylogenetic studies have used sequences from the cluster of tandemly repeated rRNA gene. Interspersed between the highly conserved structural regions coding for the 5.8S, 18S and 28S rRNA genes, are the variable internal transcribed spacer regions – ITS (Mitchell et al., 1995; Palumbi, 1996). PCR amplification of rDNA-ITS regions and the rRNA genes is possible using a set of universal primers. The variability observed in rDNA-ITS region have been used for intra- or inter-specific divergence analysis in several fungal species (Nazar et al., 1991; Liu and Sinclair, 1992; Kusaba and Tsuge, 1995; Bunting et al., 1996; Cooke and Duncan, 1997; Fouly et al., 1997; Kropp et al., 1997).

Tisserat et al. (1994) made selective primers derived from ITS sequences of *Ophiosphaerella korrae* and *O. herpotricha* for rapid diagnoses of turfgrass diseases. Liu and Sinclair (1992) identified five groups of variability in *Rhizoctonia solani* anastomosis group 2 isolates using RFLP-ITS of the rDNA. They suggested that mutation at the ITS region was the cause of evolution of these groups. Bunting et al. (1996) analyzed the rDNA ITS-1 region of *Magnaporthe* spp and observed greater variability within *M. grisea* than within *M. poe* and *M. rhizophila* isolates.

PCR amplification of ITS region of rDNA has been used to characterize variability in *C. gloeosporioides* (Mills et al., 1992). Sreenivasaprasad et al. (1992) suggested the use of rDNA-ITS 1 region to assess the molecular variability within and between isolates of *C. acutatum*, *C. gloesporioides* and *C. fragariae*. Isolates of *C.*

fragariae were separated into two groups by homology analyses of rDNA-ITS sequenced regions, while isolates from *C. gloesporioides* formed a distinct monomorphic group. Analysis of sequences of the ITS1 region of *Colletotrichum* spp showed *C. gloesporioides, C. musae* and *C. fructigenum* clustered along with *C. acutatum* (Sreenivasaprasad et al., 1994). Sherriff et al. (1994) studied the relationships within the genus *Colletotrichum* using the PCR amplification of rDNA-ITS regions. Their results showed a cluster formed by *C. lindemuthianum, C. malvarum, C. trifolii,* and *C. orbicularae*, whereas *C. gloesporioide* represented a group-species within *Colletotrichum* spp. Restriction fragment length polymorphism of both rRNA genes and the ITS-rDNA showed that *C. lindemuthianum* isolates consistently formed two main groups, but some isolates did not fall distinctly into either group (Fabre et al. 1995). The limited number of isolates used did not allow analysis of whether groupings were aligned according to host origin or virulence. Sicard et al. (1997) grouped isolates of *C. lindemuthianum* collected in Mexico, Ecuador and Argentina from wild *Phaseolus* using RFLP-ITS. Adaptation of races on cultivars of the same geographic origin was suggested because groups corresponded to three host gene pools.

Combining virulence and molecular analyses has revealed the structure of variability in *C. lindemuthianum*. In addition, the effect of geographic origin of pathogen isolates and host gene pools on variability in *C. lindemuthianum* has been demonstrated. Such coevolutionary trends should help breeding programs decide on the specific gene combinations to deploy according to local situations. The greater pathogenic diversity of *C. lindemuthianum* in the Central American countries may require control strategies where resistance genes should be deployed in a manner emphasizing resistance gene

pyramiding (Young and Kelly, 1997). In contrast, the limited variability within *C. lindemuthinaum* in North America may represent reduced pathogenicity on the commonly deployed resistance genes, resulting in an increase in the durability of resistant cultivars.

Relationship between C. lindemuthianum *and* P. vulgaris *variability.* Anthracnose has traditionally been endemic and more severe in Central America than in Andean South America or in the temperate regions of North America and Europe (Pastor-Corrales et al., 1994). Since Barrus (1918) had grouped bean cultivars according to the races alpha and beta, resistance to anthracnose became a major objective in breeding programs worldwide. The Andean *Co-1* (A) gene was the first major gene utilized to develop anthracnose resistant cultivars in common bean (Burkholder, 1918). Prior to 1973, *Co-1* gene was used as the only source of resistance in navy beans (Sanilac and Seafarer) in Michigan and Ontario. After appearance of delta race in Ontario in 1978 (Tu, 1988), the *Co-2* gene became the main source of anthracnose resistance in North America. However, identification of race 73 in Michigan and race alpha-Brazil in Ontario limited its utilization (Kelly et al., 1994; Tu, 1994) and the pyramiding of *Co-1* and *Co-2* genes was suggested as the best protection against all known races in North America (Kelly et al., 1994; Young and Kelly, 1996).

The *Co-2* (*Are*) gene, characterized in a black bean from Venezuela (Mastenbroek, 1960), was the predominant source of resistance used worldwide (Kruger et al., 1977; Fouilloux, 1979). The appearance of races virulent to the *Co-2* gene (kappa, iota, and alpha-Brazil) emphasized the need for new sources of resistance. European and

American cultivars were reported as resistance sources against these races (Kruger et al., 1977; Fouilloux, 1979). The resistance genes *Mexique I*, *II* and *III* were characterized in France from a Mexican germplasm collection (Fouilloux, 1979). The genes *Mexique II* and *III* conferred resistance to the races virulent to the gene *Co-2*, whereas *Mexique I* was susceptible to the race alpha-Brazil. Screening of 13,000 accessions for resistance to Latin American and European isolates of *C. lindemuthianum* showed that Latin American isolates were more virulent than European isolates (Schwartz et al., 1982). In addition, only 0.25% of the germplasm was resistant to all isolates. The cultivars TU, AB 136, G 2333, G 2338, G 3991, and G 4032 were resistant to nine races of *C. lindemuthianum* from Brazil (Balardin et al., 1990). G 2333 was resistant to mixtures of isolates from Middle American and Andean South American countries based on the inoculation of 20,144 accessions in CIAT (Pastor-Corrales et al., 1995).

Resistance based on major genes has been ineffective when resistance genes are used one at a time (Duvick, 1996). Since monitoring virulence in *C. lindemuthianum* has been disseminated among countries, evolving races have continually overcome resistant germplasm. Sources of resistance widely used in many breeding programs in Europe (Mastenbroeck, 1960; Goth and Zaumeyer, 1965; Fouilloux, 1979), North America (Tu and Aylesworth, 1980; Tu, 1988) and South America (Menezes and Dianese, 1988; Balardin et al., 1990) were overcome by races in different countries. The cultivars TO, PI 207262, and Mexico 222, reported resistant to European and Latin American isolates (Kruger et al., 1977; Fouilloux, 1979; Schwartz et al., 1982), were susceptible to races identified in Brazil (Menezes and Dianese, 1988). In Mexico, Garrido (1986) and Rodriguez-Guerra (1991) reported 32 different races virulent to the cultivars Cornell 49-

242, Mexico 222, PI 207262, TO, TU, and AB 136. In Nicaragua, Rava et al. (1993) identified isolates that overcame resistance in the cultivars PI 207262, TO, TU, and AB 136. The most extensive variability of *C. lindemuthianum* in Latin American countries might be influenced by greater genetic variability in the local hosts. As observed, major gene resistance has not been effective. Thus, pyramiding major resistance genes may be an appropriate breeding strategy for long-term resistance in *Phaseolus* (Young and Kelly, 1997). Resistance in G 2333 was demonstrated to be conditioned by the genes *Co-4*^{2}, *Co-5*, and *Co-7* (Young and Kelly, 1997; Young et al., 1997). In addition, knowledge of gene complementarity has been suggested to improve the efficiency of pyramiding genes for durable resistance (Duvick, 1996). A resistance gene combination must be deployed until it is vulnerable only to virulence combinations that do not occur in the pathotype array (Casela et al., 1996).

Morphological, biochemical and molecular markers (Gepts, 1988; Singh et al., 1991) have been used to demonstrate the existence of Middle American and South American Andean gene pools in *P. vulgaris*. If pathogens were specialized on one of the host gene pools, then transferring resistance genes between gene pools may provide a more durable resistance (Gepts, 1988; Singh et al., 1991). In addition, the reduced virulence of isolates from the Andean region compared to isolates from Middle American regions suggests that deployment of resistance genes between gene pools will improve durable resistance (Gepts, 1988; Singh et al., 1991; Young and Kelly, 1997). Pathogen congruence with the two *P. vulgaris* gene pools has been demonstrated. *Uromyces phaseoli* (Stavely, 1982; Stavely, 1984; Maclean, 1995) and *Phaeoisariopsis griseola* (Guzman et al., 1995) appear to be grouped according to *P. vulgaris* gene pools. A

previous study of twelve *C. lindemuthianum* isolates found that RAPD and RFLP markers divided the species into two groups (Fabre et al., 1995). The groups suggested some specialization within *C. lindemuthianum* that corresponded to the two gene pools within *P. vulgaris*. Pastor-Corrales (1996) reported that *C. lindemuthianum* can be divided into two groups based on virulence; one specializing on hosts from the Middle American gene pool and the second specializing on Andean hosts. Using RFLP-ITS, Sicard et al. (1997) grouped isolates of *C. lindemuthianum* collected in Mexico, Ecuador and Argentina from wild *Phaseolus*. Two groups of races were observed in Mexico, corresponding to the Middle American and Andean diversity center. Races from Ecuador and Argentina both corresponding to the Andean gene pool, were monomorphic. The information on variability in *C. lindemuthianum* and the specialization of specific races on one of the host gene pools obtained from virulence and molecular analyses, may be valuable in breeding for resistance. The process of pyramiding genes could be improved through marker assisted selection (MAS) if molecular markers linked tightly to the resistance genes were available (Kelly et al., 1994). In this case if direct selection for a phenotype is not practical or feasible due to epistasis, MAS can accelerate the breeding process and be used to identifiy uncharacterized resistance genes for which no race is available (Young et al. 1997).

Conclusions. Variability in *C. lindemuthianum* has been characterized worldwide. Unfortunately local codes and different differential series have biased the structuring of broader pathogen populations. A standard differential series was proposed, but an imbalance among cultivars from *P. vulgaris* gene pools might favor identification of

races belonging to the Middle American reaction group. The multigenic resistance in other cultivars such as G 2333 might select races with multiple avirulence genes. Thus, information regarding the gene-for-gene relationship in the *C. lindemuthianum* - *P. vulgaris* pathosystem would be biased. Developing durable resistance to *C. lindemuthianum* must be based on reliable characterization of variability in this pathogen. Neutral molecular markers are less influenced by changes in the environment and have been more informative in assessing variability. RAPDs can be used to identify, group, and relate pathogen isolates to geographic or host factors. However, problems related with RAPDs have indicated the need for markers, which consistently reproduce the variability within the genome of a pathogen. The analysis of the variable rDNA-ITS regions have been used for phylogenetic analyses at inter- or intra-species level in *Colletotrichum*. Since *C. lindemuthianum* variability has consistently been characterized, breeding programs might be able to identify new sources of resistance for their local needs. Resistance genes could be intelligently pyramided and segregating populations adequately screened either directly or indirectly using MAS. Deployment of gene combinations might be planned according to specific geographic regions resulting in a more durable resistance to *C. lindemuthianum*.

General objectives. Characterization of variability in *C. lindemuthianum* has been considered a crucial step to help control anthracnose using genetic resistance. Understanding of *C. lindemuthianum* population structure would assist breeding programs to select and deploy resistance genes according to specific situations. The objectives of this work were: *a.* characterization of diversity in *C. lindemuthianum* from isolates collected in South, Central, and North American countries using virulence and

molecular (RAPD) polymorphism; *b.* sequence homology and RFLP analysis of rDNA-ITS regions of *C. lindemuthianum* isolates; and, *c.* genetic analyses of virulence and resistance in the *P. vulgaris* – *C. lindemuthianum* pathosystem. The adequacy of molecular markers in establishing phylogenetic relationships, the correlation of molecular markers with race structure and geographic structure, the structure of *C. lindemuthianum* populations, and the effect of host diversity on pathogen variability will be discussed.

LIST OF REFERENCES

Andrus, C. F., and Wade, B. L. 1942. The factorial interpretation of anthracnose resistance in beans. Pages 1-29: in Tech. Bull. No. 310. USDA. Washington.

Aufauvre-Brown, A., Cohen, J., and Holden, D. W. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. Journal of Clinical Microbiology 30:2991-2993.

Balardin, R., and Kelly, J. D. 1996. Identification of Race 65-epsilon of bean anthracnose (*Colletotrichum lindemuthianum*) in Michigan. Plant Dis. 80:712.

Balardin, R. S., Pastor-Corrales, M. A., and Otoya, M. M. 1990. Variabilidade patogênica de *Colletotrichum lindemuthianum* no Estado de Santa Catarina. Fitopatol. Bras. 15:243-245.

Bannerot, H. 1965. Results de l'infection d'une collection de haricots par six races physiologiques d'anthracnose. Annales de Amelioration des Plantes (Paris) 15:201-222.

Barrus, M. F. 1911. Variation of varieties of beans in their susceptibility to anthracnose. Phytopathology 1:190-195.

Barrus, M. F. 1918. Varietal susceptibility of beans to strains of *Colletotrichum lindemuthianum* (Sacc. & Magn.) B. & C.. Phytopathology 8:589-605.

Blondet, A. 1963. L'anthracnose du haricot: etudé des races physiologiques du *Colletotrichum lindemuthianum*. Ph.D. thèse, Faculté de Sciense, Paris, France.

Bruns, T. D., White, T. J., and Taylor, J. N. 1991. Fungal Molecular systematic. Annual Review of Ecology and Systematic 22:525-564.

Bunting, T. E., Plumley, K. A., Clarke, B. B., and Hillman, B. I. 1996. Identification of *Magnaporthe poae* by PCR and examination of its relationship to other fungi by analysis of their nuclear rDNA ITS-1 regions. Phytopathology 86:398-404.

Burkholder, W. H. 1923. The gamma strain of *Colletotrichum lindemuthianum* (Sacc. et Magn.) B.et C.. Phytopathology 13:316-323.

Casela, C. R., Ferreira, A. S., Zeller, K. A., and Levy, M. 1995. Pathotype variation in the sorghum anthracnose fungus: a phylogenetic perspective for resistance breeding. Pages 257-288 in: Disease analysis through genetics and biotechnology. J. F. Leslie and R. S. Frederiksen, eds. Iowa State University Press. Ames, IO.

Charrier, A., and Bannerot, H. 1970. Contribution à l'étude des races phisiologiques de l'anthracnose du haricot. Ann. Phytopathol. 2:489-506.

Cooke, D. E. L. and Duncan, J. M. 1997. Phylogenetic analysis of *Phytophthora* species baed on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycol. Res. 101:667-677.

Crowhurst, R. N., Hawthorne, B. T., Rikkerink, E. H. A., and Templeton, M. D. 1991. Differentiation of *Fusarium solani* f.sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Curr. Genet. 20:391-396.

Cruickshank, I. A. M. 1966. Strains of *Colletotrichum lindemuthianum* (Sacc. and Magn.) in eastern Australia. J. Aust. Inst. Agric. Sci. 32:134-135.

Drifhout, E., and Davis, J. H. C. 1989. Selection of a new set of homogeneously reacting bean (*Phaseolus vulgaris*) differentials to differentiate races of *Colletotrichum lindemuthianum*. Plant Pathology 38:391-396.

Dillard, H. R., and Cobb, A. C. 1993. Survival of *Colletotrichum lindemuthianum* in bean debris in New York State. Plant Dis. 77:1233-1238.

Duvick, D. N. 1996. Plant Breeding, an evolutionary concept. Crop Sci. 36:539-548.

Fabre, J. V., Julien, J., Parisot, D., and Dron, M. 1995. Analysis of diverse isolates of *Colletotrichum lindemuthianum* infecting common bean using molecular markers. Mycol. Res. 99:429-435.

Fouilloux, G. 1975. L'anthracnose du haricot: étude des relations entre les pathotypes anciens et nouveaux; étude de nouvelles sources de résistance "totale". Pages 81-92 in: Réunion Eucarpia Haricot, Versailles, France. Centre National de Recherches Agronomiques, Paris, France.

Fouilloux, G. 1979. New races of bean anthracnose and consequences on our breeding programs. Pages 221-235 in: Disease of tropical food crops. H. Maraite and J.A. Meyer, eds. Louvain-la-Neuve, Belgium.

Fouly, H. M., Wilkinson, H. T., and Chen, W. 1997. Restriction analysis of internal trascribed spacers and the samll subunit gene of ribosomal DNA among four *Gaeumannomyces* species. Mycologia 89:590-597.

Garrido, E. R. 1986. Identificación de razas fisiológicas de *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scrib. en Mexico y busqueda de resistência genética a este hongo. MSc thesis, Institución de Ensenanza y Investigación en Ciencias Agrícolas, Montecillos, Mexico.

Gepts, P. 1988. A Middle American and an Andean common bean gene pool. Pages 375-390 in: Genetic resources of *Phaseolus* beans; their maintenance, domestication, and utilization. P. Gepts, ed. Kluwer. London.

Goodwin, P. H., and Annis, S. L. 1991. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Applied and Environmental Microbiology 27:2482-2486.

Goth, R. W., and Zaumeyer, W. J. 1965. Reactions of bean varieties to four races of anthracnose. Plant Dis. Rep. 49:815-818.

Guthrie, P. A. I., Magill, C.W., Frederiksen, R. A., and Odvody, G. N. 1992. Random amplified polymorphic DNA markers: a system for identifying and differentiating isolates of *Colletotrichum graminicola*. Phytopathology 82:832-835.

Guzman, P., Donado, M. R., and Galvez, G. E. 1979. Perdidas economicas causadas por la antracnosis del frijol *Phaseolus vulgaris* en Colombia. Turrialba 29:65-67.

Guzman, P., Gilbertson, R. L., Nodari, R., Johnson, W. C., Temple, S. R., Mandala, D., Mkandawire, A. B. C., and Gepts, P. 1995. Characterization of variability in the fungus *Phaeisariopsis griseola* suggests coevolution with the common bean (*Phaseolus vulgaris*). Phytopathology 85:600-607.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K., and Zimmer, E. A. 1996. Nucleic acids IV: sequencing and cloning. Pages 321-381 in: Molecular Systematics. D.M. Hills, C. Moritz, and B.K. Mable, eds. Sinauer, Sunderland, MA.

Hubbeling, N. 1976. Selection for resistance to anthracnose, particularly in respect to the "ebnet" race of *Colleotrichum lindemuthianum*. Ann. Rep. Bean Improv. Coop. 19:49-50.

Hubbeling, N. 1977. The new jota race of *Colletotrichum lindemuthianum*. Ann. Rep. Bean Improv. Coop. 20:58.

Kelly, J. D., Afanador, L., and Cameron, L. S. 1994. New races of *Colletotrichum lindemuthianum* in Michigan and implications in dry bean resistance breeding. Plant Dis. 78:892-894.

Kimati, H. 1966. Algumas raças fisiológicas de *Colletotrichum lindemuthianum* (Sac. et Magn.) Scrib. que ocorrem no Estado de São Paulo. An. Esc. Super. Agric. Luiz de Queiróz (Sao Paulo) 23:411-437.

Kolmer, J. A., Liu, J. Q., and Sies, M. 1995. Virulence and molecular polymorphism in *Puccinia recondita* f.sp. *tritici* in Canada. Phytopathology 85:276-285.

Kropp, B. R., Hansen, D. R., Wolf, P. G., Flint, K. M., and Thomson, S. V. 1997. A study on the phylogeny of the Dyer's woad rust fungus and other species of *Puccinia* from crucifers. Phytopathology 87:565-571.

Kusaba, M., and Tsuge, T. 1995. Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal trascribed spacers of ribosomal DNA. Curr Genet 28:491-498.

Kruger, J., Hoffmann, G. M., and Hubbeling, N. 1977. The kappa race of *Colletotrichum lindemuthianum* and sources of resistance to anthracnose in *Phaseolus* beans. Euphytica 26:23-25.

Kusaba, M., and Tsuge, T. 1995. Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal trascribed spacers of ribosomal DNA

Leakey, C. L. A., and Simbwa-Bunnya, M. 1972. Races of *Colletotrichum lindemuthianum* and implications for bean breeding in Uganda. Ann. Appl. Biol. 70:25-34.

Liu, Z. L., and Sinclair, J. B. 1992. Genetic diversity of *Rhizoctonia solani* anastomosis group 2. Phytopathology 82:778-787.

Maclean, D. J., Braithwaite, K.S., Irwin, J.A.G., Manners, J.M., and Groth, J.V. 1995. Random Amplified Polymorphic DNA reveals relationships among diverse genotypes in Australian and American collections of *Uromyces appendiculatus*. Phytopathology 85:757-765.

Mastenbroeck, C. 1960. A breeding program for resistance to anthracnose in dry shell haricot beans, based on a new gene. Euphytica 9:177-184.

McDonald, B. A., and McDermott, J. M. 1993. Population genetics of plant pathogenic fungi. BioScience 43:311-319.

McDonald, B. A. 1997. The population genetics of fungi: tools and techniques. Phytopathology 87:448-453.

Megnegneau, B., Debets, F., and Hoekstra, R. F. 1993. Genetic variability and relatedness in the complex group of black *Aspergilli* based on random amplification of polymorphic DNA. Curr Genet 23:323-329.

Menezes, J. R., and Dianese, J. C. 1988. Race characterization of Brasilian isolates of *Colletotrichum lindemuthianum* and detection of resistance to anthracnose in *Phaseolus vulgaris*. Phytopathology 78:650-655.

Michelmore, R. W., and Hulbert, S. H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol. 25:383-404.

Milgroom, M. G. 1995. Analysis of population structure in fungal plant pathogens. Pages 213-229 in: Disease analysis through genetics and Biotechnology. J. F. Leslie, and R.A. Frederiksen, eds. Iowa State University Press. Ames, IO.

Mills, P. R., Sreenivasaprasad, S., and Brown, A.E. 1992. Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. FEMS Microbiology Letters 98:137-144.

Mitchell, J. I., Roberts, P. J., and Moss, S. 1995. Sequence or structure? A short review on the application of nuclei acid sequence information to fungal taxonomy. Mycologist 9:67-75.

Mujica, R. F. 1952. Razas fisiológicas y susceptibilidad varietal de los frijoles chilenos a la antracnosis. Agri. Téc. (Santiago) 12:37-45.

Nazar, R. N., Hu, X., Schmidt, J., Culham, D., and Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. Physiol. and Mol. Plant Pathol. 39:1-11.

Nicholson, P., and Rezanoor, H. N. 1994. The use of random amplified polymorphic DNA to identify pathotype and detect variation in *Pseudocercosporella hertrichoides*. Mycol. Res. 98:13-21.

Noyola, I. T. U., Lopez, G. F., and Muruaga, J. 1984. Determinación de razas fisiológicas de *Colletotrichum lindemuthianumm* (Sacc. & Magn.)Bri & Cav. Resúmenes del XI Cong. Nac. de Fitopatología Soc. Mexicana de Fitopatología, A.C. San Luis Potosí, Mexico.

Oliari, L., Vieira, C., and Wilkinson, R. E. 1973. Physiologic races of *Colletotrichum lindemuthianum* in the state of Minas Gerais, Brazil. Plant Dis. Rep. 57:870-872.

Palumbi, S. R. 1996. Nucleic acids II: the polymerase chain reaction. Pages 205-247 in: Molecular Systematics. D. M. Hills, C. Moritz, and B. K. Mable, eds. Sinauer, Sunderland, MA.

Pastor-Corrales, M. A (ed.). 1988. La antracnosis del frijol comum, *Phaseolus vulgaris*, en America Latina. Documento de Trabajo no. 113. CIAT. Cali, Colombia.

Pastor-Corrales, M. A. 1991. Estandarización de variedades diferenciales y de designación de razas de *Colletotrichum lindemuthianum*. Phytopathology 81:694 (abstract).

Pastor-Corrales, M. A. 1996. Traditional and molecular confirmation of the coevolution of beans and pathogens in Latin America. Ann. Rep. Bean Improv. Coop. 39:46-47.

Pastor-Corrales, M. A., and Tu, J. C. 1989. Anthracnose. Pages 77-104 in: Bean production problems in the tropics. H. F. Schwartz, and M. A. Pastor-Corrales, eds. CIAT. Cali, Colombia.

Pastor-Corrales, M. A., Erazo, O.A., Estrada, E. I., and Singh, S. P. 1994. Inheritance of anthracnose resistance in common bean accession G 2333. Plant Dis. 78:959-962.

Pastor-Corrales, M. A., Otoya, M. M., and Molina, A. 1995. Resistance to *Colletotrichum lindemuthianum* isolates from Middle America and Andean South America in different common bean races. Plant Dis. 79:63-67.

Peurer, H. 1931. Continued investigation on the occurrence of biological strains in *Colletotrichum lindemuthianum* (Sacc. & Magn.) Bri. et Cav. Phytopathologische Zaeitshrit 4:83-112.

Pio-Ribeiro, G., and Chaves, G. M. 1975. Raças fisiológicas de *Colletotrichum lindemuthianum* (Sacc. et Magn.) Scrib. que ocorrem em alguns municípios de Minas Gerais, Espirito Santo e Rio de Janeiro. Experientiae 19:95:118.

Rava, C. A., Molina, J., Kauffmann, M., and Briones, I. 1993. Determinación de razas fisiológicas de *Colletotrichum lindemuthianum* en Nicaragua. Fitopatol. Bras. 18:388-391.

Restrepo, S. 1994. DNA polymorphism and virulence variation of *Colletotrichum lindemuthianum* in Colombia. M.Sc. thesis. Universite Paris IV, Paris-Grignon, France.

Rodriguez, R. 1991. Identificación de razas patogenicas de *Colletotrichum lindemuthianum* (Sacc. y Magn.) Scrib. en el estado de Durango mediante un sistema propuesto internacionalmente y respuesta de genótipos de frijol tolerantes a sequia a razas del patógeno. M.Sc., Parasitologia Agricola, Universidad Autonoma Agraria "Antonio Narro", Buenavista, Mexico.

Schnock, M. G., Hoffmann, G. M., and Kruger, J. 1979. A new physiological strain of *Colletotrichum lindemuthianum* infecting *Phaseolus vulgaris* L. HortScience 10:140.

Schreiber, F. 1932. Resistenzzuchtung bei *Phaseolus vulgaris*. Phytopathol. Z. 4:415-454.

Schwartz, H. F., Pastor-Corrales, M. A., and Singh, S. P. 1982. New sources of resistance to anthracnose and angular leaf spot of beans (*Phaseolus vulgaris*). Euphytica 31:741-754.

Sicard, D., Michalakis, Y., Dron, M., and Neema, C. 1997. Genetic diversity and pathogenic variation of *Colletotrichum lindemuthianum* in the three centers of diversity of its host, *Phaseolus vulgaris*. Phytopathology, 87:807-813.

Singh, S. P., Gepts, P., and Debouck, D. G. 1991. Races of common bean (*Phaseolus vulgaris*, Fabaceae). Econ. Bot. 45:379-396.

Sherriff, C., Whelan, M. J., Arnold, G. M., Lafay, J. F., Brygoo, Y., and Bailey, J. A. 1994. Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. Experimental Mycology 18:121-138.

Smith, J. J., Scott-Craig, J. S., Leadbetter, J. R., Bush, G. L., Roberts, D. L., and Fulbright, D. W. 1995. Characterization of random amplified polymorphic DNA (RAPD) products from *Xanthomonas campestris*: implications for the use of RAPD products in phylogenetic analysis. Mol. Phylogenet. Evol. 3:135-145.

Sreenivasaprasad, S., Brown, A. E., and Mills, P. R. 1992. DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. Physiol. and Mol. Plant Pathol. 41:265-281.

Sreenivasaprasad, S., Mills, P. R., and Brown, A. E. 1994. Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. Mycol. Res. 98:186-188.

Stavely, J. R. 1982. The potential for controlling bean rust by host resistance. Ann. Rep. Bean Improv. Coop. 25:28-30.

Stavely, J. R. 1984. Pathogenic specialization in *Uromyces phaseoli* in the United States and rust resistance in beans. Plant Dis. 68:95-99.

Sutton, B. C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. Pages 1-26 in: *Colletotrichum*-Biology, Pathology and Control. J.A. Bailley and M.J. Jeger, eds. C.A.B. International, Wallingford, UK.

Tisserat, N. A., Hulbert, S. H., and Sauer, K. M. 1994. Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. Phytopathology 84:478-482.

Tu, J. C. 1988. Control of bean anthracnose caused by delta and lambda races of *Colletotrichum lindemuthianum* in Canada. Plant Dis. 72:5-7.

Tu, J. C. 1992. *Colletotrichum lindemuthianum* on bean. Population dynamics of the pathogen and breeding for resistance. Pages 203-224 in: *Colletotrichum*-Biology, Pathology and Control. J. A. Bailley and M. J. Jeger, eds. C.A.B. International, Wallingford, UK.

Tu, J. C. 1994. Occurrence and characterization of the alpha-Brazil race of bean anthracnose (*Colletotrichum lindemuthianum*) in Ontario. Can. J. Plant Pathol. 16:129-131.

Tu, J. C., and Aylesworth, J. W. 1980. An effective method for screening white (pea) bean seedlings (*Phaseolus vulgaris* L.) for resistance to *Colletotrichum lindemuthianum*. Phytopathol. Z. 99:131-137.

Tu, J. C., Sheppard, J. W., and Laidlaw, D. M. 1984. Occurrence and characterization of the epsilon race of bean anthracnose in Ontario. Plant Dis. 68:69-70.

Vilarinhos, A. D., Paula Jr., T. J., de Barros, E. G., and Moreira, M. A. 1995. Characterization of races of *Colletotrichum lindemuthianum* by the random amplified polymorphic DNA technique. Fitopatol. Bras. 20:194-198.

Waterhouse, W. L. 1955. Studies of bean anthracnose in Australia. Proc. Linn. Soc. N.S.W. 80:71-83.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531-6535.

Yerkes, W. D. Jr. 1958. Additional new races of *Colletotrichum lindemuthianum* in Mexico. Plant Dis. Rep. 42:329.

Yerkes, W. D., and Teliz-Ortiz, M. 1956. New races of *Colletotrichum lindemuthianum* in Mexico. Phytopathology 46:564-567.

Young, R. A., and Kelly, J. D. 1996. Characterization of the genetic resistance to *Colletotrichum lindemuthianum* in common bean differential cultivars. Plant Dis. 80:650-654.

Young, R. A., and Kelly, J. D. 1997. RAPD markers linked to three major anthracnose resistance genes in common bean. Crop Sci. 37:940-946.

Young, R. A., Melotto, M., Nodari, R. O., and Kelly, J. D. 1997. Marker assisted dissection of oligogenic anthracnose resistance in the common bean cultivar, G2333. Theor. Appl. Genet. (in press).

Zaumeyer, W. J., and Thomas, H. R. 1957. A monographic study of bean diseases and methods for their control. USDA. Agr. Tech. Bull. No. 868. 255p.

Chapter 1

VIRULENCE AND MOLECULAR DIVERSITY IN *COLLETOTRICHUM LINDEMUTHIANUM* FROM SOUTH, CENTRAL AND NORTH AMERICA

ABSTRACT

One hundred thirty-eight isolates of *Colletotrichum lindemuthianum* from Argentina, Brazil, the Dominican Republic, Honduras, Mexico and the United States were characterized into 41 races based on virulence to twelve differential cultivars of *Phaseolus vulgaris*. These 41 races were categorized into two groups, those found over a wide geographic area and those restricted to a single country. Races 7, 65, and 73 were widespread. Race 73 was the most common (28%). Race 7 was found once in Argentina, and Mexico, but at higher frequency in the United States. Race 65 was found repeatedly in Brazil and the United States. Although 39% of the races were detected multiple times and three races were widespread, no race was isolated from both *P. vulgaris* gene pools. Phenetic analyses showed no obvious patterns correlated with virulence clusters. No geographic patterning was evident. Molecular polymorphism revealed by random amplified polymorphic DNA (RAPD) confirmed the extensive variability of *C. lindemuthianum*. Virulence phenotypes were grouped into 15 clusters. The two largest clusters contained isolates from all geographic regions sampled. Molecular polymorphism was observed among isolates from races 65, and 73 within and among countries, except among Brazilian isolates of race 65. Genetic diversity of *C.*

lindemuthianum was shown to be the greatest in Mexico and Honduras. Our data suggest that *C. lindemuthianum* may not be highly structured to specific *Phaseolus* gene pools.

INTRODUCTION

Colletotrichum lindemuthianum (Sacc. & Magnus) Lams. –Scrib. causes anthracnose of common bean (*Phaseolus vulgaris* L.). The disease is found worldwide wherever common beans are grown, but is especially important in sub-tropical and temperate region (Pastor-Corrales and Tu, 1989). Yield losses can be as high as 90% or more in some years (Zaumeyer and Thomas, 1957). Because cost-effective chemical control is lacking, host resistance has traditionally been used to control anthracnose, despite the high variability in *C. lindemuthianum* (Andrus and Wade, 1942; Barrus, 1918; Blondet, 1963; Burkholder, 1923; Fouilloux, 1979; Kruger et al., 1977; Menezes and Dianese, 1988; Waterhouse, 1955). A standardized binary nomenclature system based on a set of twelve differential cultivars has been developed to characterize virulence (Pastor-Corrales, 1991). Using this system 38 races were reported in Mexico (Garrido, 1986; Rodrigues-Guerra, 1991); 7 races were identified in a group of 10 isolates from Nicaragua (Rava et al., 1993); 33 races were characterized from a group of 178 isolates from Colombia (Restrepo, 1994), and 3 races were described in the United States (Balardin and Kelly, 1996; Kelly et al., 1994) Since only a few races are extremely widespread, it is not presently known if the virulence pattern of *C. lindemuthianum* is due to repeated evolution of a race or efficient seed-borne dispersal between areas (Pastor-Corrales and Tu, 1989).

Virulence in common bean pathogens, *Uromyces appendiculatus* (Maclean et al., 1995) and *Phaeoisariopsis griseola* (Guzman et al., 1995), appears to be highly structured within each species. Patterns generated using randomly amplified polymorphic

(RAPD) DNA markers indicated that *U. appendiculatus* was highly structured geographically (Maclean et al., 1995). RAPD patterns divided *P. griseola* isolates into two distinct groups; one group was specialized on *P. vulgaris* hosts of Andean ancestry, while the second group was obtained from common bean hosts of Middle American origin (Gepts, 1988, Singh et al., 1991). This grouping of fungal races congruently with host gene pools has been interpreted as evidence for coevolution (Guzman et al., 1995; Pastor-Corrales, 1996; Singh et al., 1991). It also suggests combining complementary genes to exclude all pathogen lineages (Casela et al., 1995; Duvick, 1996; Levy et al., 1993; Young and Kelly, 1997) where resistance genes from Middle American hosts can be utilized effectively in Andean hosts and vice versa. Indeed, virulence within common bean rust (*Uromyces phaseoli*) has been shown to be specialized onto either Andean or Middle American germplasm (Maclean et al., 1995; Stavely, 1982; Stavely, 1984), leading some authors to advocate lineage based breeding to control this disease (Gepts, 1988) and other common bean pathogens, including *C. lindemuthianum* (Singh et al., 1991; Young and Kelly, 1997).

A study that utilized RAPD markers, restriction fragment length polymorphism of both nuclear ribosomal genes and the internal transcribed spacer region of the ribosomal subunit found that *C. lindemuthianum* isolates consistently formed two main groups, but some isolates did not fall distinctly into one or the other group (Fabre et al., 1995). The limited number of isolates used did not allow analysis of whether groupings were aligned according to origin of host or virulence. A similar study conducted with isolates of *C. lindemuthianum* collected from wild *Phaseolus* hosts in Mexico, Ecuador and Argentina showed groupings of isolates corresponding to Andean and Middle American host gene

pools (Sicard et al., 1997). In this study we sought to determine how variability within *C. lindemuthianum* is structured. Isolates were collected from both Andean and Middle American *Phaseolus* genotypes grown in Argentina, Brazil, Canada, the Dominican Republic, Honduras, Mexico and the United States. Virulence analyses and RAPD polymorphism were used separately to group isolates using cluster analyses.

MATERIALS AND METHODS

Colletotrichum lindemuthianum *isolates.* One hundred thirty-eight conidial isolates were collected between 1992 and 1994 from common bean cultivars grown in Argentina, Brazil, the Dominican Republic, Honduras, Mexico and the United States (Table 1.1, Appendix A1.1). The fungus was isolated from diseased leaves or pods showing characteristic anthracnose symptoms (Pastor-Corrales and Tu, 1989). Small pieces of infected tissue were surface-sterilized and incubated on petri dishes containing modified Mathur's culture medium. The culture medium was prepared with dextrose (8 $g.l^{-1}$), $MgSO_4.7H_2O$ (2.5 $g.l^{-1}$), KH_2PO_4 (2.7 $g.l^{-1}$), neopeptone (2.4 $g.l^{-1}$), yeast extract (2.0 $g.l^{-1}$), and agar (16 $g.l^{-1}$). Plant tissue was incubated in complete darkness at 24°C for 7 days or until formation of acervuli morphologically resembling *C. lindemuthianum* (Pastor-Corrales and Tu, 1989). Spore suspensions for seedling inoculation were prepared from purified single-conidial isolates by flooding plates with 5 ml of 0.01% Tween 80 in distilled water. Spores were dislodged by scraping the culture surface with a spatula, filtering through cheesecloth, and the concentration was adjusted to 1.0×10^6 $spores.ml^{-1}$ with a hemocytometer.

Determination of virulence phenotypes. Races of *C. lindemuthianum* were characterized based on virulence of the 138 isolates on the differential *P. vulgaris* series proposed by Pastor-Corrales (1991). Seeds for each of the twelve cultivars were planted in flats containing Baccto planting mix (Michigan Peat Co., Houston TX), and grown under greenhouse conditions (16-h day length at 25°C), for 7 to 10 days until seedlings had reached the primary leaf stage. Six to 10 seedlings were spray-inoculated with standardized spore suspensions of each isolate of *C. lindemuthianum*. Suspensions were applied until runoff on the stem and to both surfaces of the unifoliolate leaves. After inoculation, plants were maintained in high humidity (>95%) for 48 h at 22 to 25°C. Plants were allowed to dry and were then transferred to greenhouse benches for 5 days before disease symptom evaluation. Seven days after inoculation, seedlings were rated for disease reaction based on a 1 to 9 severity scale (Balardin et al., 1990). Disease reactions were recorded as resistant (grade 1 to 3) for those plants with no visible disease symptoms or only a few, very small lesions mostly on primary leaf veins. Plants with numerous enlarged lesions or with sunken cankers on the lower sides of leaves or hypocotyls were recorded as susceptible (grade 4 to 9). Inoculations were repeated at least twice for each isolate. The identified races were assigned a value using the binary nomenclature system (Pastor-Corrales, 1991). Each differential cultivar has an assigned number (2^n) where n corresponded to the place occupied by the cultivar within the differential series (Appendix A1.2). The designation of a race number was obtained by summing the 2^n values of all cultivars exhibiting susceptible reaction to the isolate being inoculated.

Table 1.1. - Race designation, number of isolates, and reaction of common bean differential cultivars (+, susceptible and -, resistant) of 41 races of *C. lindemuthianum* identified from 138 isolates collected on *Phaseolus* hosts from Middle American and Andean gene pools grown in the listed countries.

	Differential Cultivars and known resistance genes[b]												
Race[a]	A	B[d] *Co-1*	C[d]	D *Co-2*	E	F[d]	G *Co-3*	H	I *Co-4*	J *Co-5*	K *Co-6*	L *Co-4²/Co-5/Co-7*	Isolates[c]
1	+	-	-	-	-	-	-	-	-	-	-	-	Bra(M3)*
2	-	+	-	-	-	-	-	-	-	-	-	-	Mex(A1)
7	+	+	+	-	-	-	-	-	-	-	-	-	Arg(A1), Mex (A1), US(A6)*
9	+	-	-	+	-	-	-	-	-	-	-	-	Hon(M4)*
17	+	-	-	-	+	-	-	-	-	-	-	-	Bra(M-5)*
19	+	+	-	-	+	-	-	-	-	-	-	-	DR(A-1)
55	+	+	+	-	+	+	-	-	-	-	-	-	DR(A-1)*
65	+	-	-	-	-	-	+	-	-	-	-	-	Bra(M6)*, US(M13)*
73	+	-	-	+	-	-	+	-	-	-	-	-	Bra(M3)*, Hon(M4)*, Mex(M2)*, US(M29)*

Table 1.1 (cont'd)

81	+	-	-	-	+	-	+	-	-	-	-	-	**Arg(M1)**
89	+	-	-	+	+	-	+	-	-	-	-	-	**Bra(M2)***
193	+	-	-	-	-	-	+	+	-	-	-	-	**Mex(M1)**
201	+	-	-	+	-	-	+	+	-	-	-	-	**Hon(M1)***
209	+	-	-	-	+	-	+	+	-	-	-	-	**Mex(M1)**
256	-	-	-	-	-	-	-	-	+	-	-	-	**Mex(M1)***
257	+	-	-	-	-	-	-	-	+	-	-	-	**Mex(M1)***
320	-	-	-	-	-	-	+	-	+	-	-	-	**Mex(M1)**
321	+	-	-	-	-	-	+	-	+	-	-	-	**Mex(M3)***
337	+	-	-	-	+	-	+	-	+	-	-	-	**Bra(M1)**
357	+	-	+	-	-	+	+	-	+	-	-	-	**Mex(M3)***
448	-	-	-	-	-	-	+	+	+	-	-	-	**Mex(M5)**
449	+	-	-	-	-	-	+	+	+	-	-	-	**Mex(M9)***
453	+	-	+	-	-	-	+	+	+	-	-	-	**Mex(M2)***
457	+	-	-	+	-	-	+	+	+	-	-	-	**Mex(M4)***
465	+	-	-	-	+	-	+	+	+	-	-	-	**Mex(M1)**
469	+	-	+	-	+	-	+	+	+	-	-	-	**Mex(M1)**
521	+	-	-	+	-	-	-	-	-	+	-	-	**Hon(M1)**
833	+	-	-	-	-	-	+	-	+	+	-	-	**Mex(-^{e}-1)**
1033	+	-	-	+	-	-	-	-	-	-	+	-	**Hon(M1)***

1165	+	-	+	+	-	-	-	+	-	-	+	-	**Mex(-[e]-1)***
1344	-	-	-	-	-	-	+	-	+	-	+	-	**Mex(M1)**
1431	+	+	+	-	+	-	-	+	+	-	+	-	**Mex(M1)**
1472	-	-	-	-	-	-	+	+	+	-	+	-	**Mex(M2)***
1545	+	-	-	+	-	-	-	-	-	+	+	-	**Hon(M1)**
1600	-	-	-	-	-	-	+	-	-	+	+	-	**Mex(M1)**
1601	+	-	-	-	-	-	+	-	-	+	+	-	**Hon(M1)**
1673	+	-	-	+	-	-	-	+	-	+	+	-	**Hon(M2)***
1677	+	-	+	+	-	-	-	+	-	+	+	-	**Hon(M1)**
1741	+	-	+	+	-	-	+	+	-	+	+	-	**Hon(M1)***
1929	+	-	-	+	-	-	-	+	+	+	+	-	**Hon(M1)***
1993	+	-	-	+	-	-	+	+	+	+	+	-	**Hon(M4)***

[a] Race identification according to binary nomenclature system (Pastor-Corrales, 1991).

[b] Common bean differential cultivars used to characterize races of *C. lindemuthianum* followed by their respective binary value (Pastor Corrales, 1991): A (Michelite - 1), B (Michigan Dark Red Kidney - 2), C (Perry Marrow - 4), D (Cornell 49242 - 8), E (Widusa - 16), F (Kaboon - 32), G (Mexico 222 - 64), H (PI 207262 - 128), I (TO - 256), J (TU - 512), K (AB 136 - 1024), and L (G 2333 - 2047). Known *Co* resistance genes are shown (Young and Kelly, 1996; Young et al., 1997)

[c] Geographic origin of isolates: Arg (Argentina), Bra (Brazil), DR (Dominican Republic), Hon (Honduras), Mex (Mexico), and US (United States), followed in the parentheses by the host gene pool (A - Andean and M - Middle America), and the number of times the race was isolated in the country. The gene pool was determined based on host genotype identified during the sampling of isolates and supported by previous knowledge.

[d] Cultivars from the Andean gene pool.

[e] Host genotype not identified.

* Subjected to RAPDs analysis.

Determination of molecular phenotypes. Sixty single-conidial isolates of *C. lindemuthianum* were characterized using RAPD marker data. These included 28 races selected as representative of the full range of virulence phenotypes (Table 1.1), plus 32 isolates previously reported in Brazil, Canada, Colombia, the Dominican Republic, Guatemala, Mexico, the Netherlands, Peru, and the United States (Andrus and Wade, 1942; Barrus, 1918; Burkholder, 1923; Drifhout and Davis, 1989; Menezes and Dianese, 1988, Pastor-Corrales et al., 1994; Pastor-Corrales et al., 1995; Restrepo, 1994; Schwartz et al., 1982; Tu et al., 1984) but not found in this survey (Table 1.2).

DNA extraction of *C. lindemuthianum* races followed the protocol modified from Edwards et al. (1991). Mycelium of each race, grown in petri dishes containing 20 ml of liquid potato-dextrose medium (20% potato) and 240 µl ampicillin solution (25 $mg.ml^{-1}$ in 70% EtOH), was used for DNA extraction. Each petri dish was inoculated with 4 plugs of one single-conidial race, incubated for 7 days at 22 to 25°C in darkness without shaking. Mycelium was harvested, surface-dried, lyophilized for 36 h, and stored at -20°C. Dried mycelium was ground to a fine powder using a precooled sterile mortar and pestle. Microcentrifuge tubes (1.5 ml) were filled with 260 mg of ground mycelium which was dispersed in 400 µl of hot (65°C) 2x CTAB extraction buffer (2% CTAB, 100 mM Tris Base, 10 mM EDTA, 0.7 M NaCl, pH 7.0). Four hundred µl of phenol:chloroform:isoamyl alcohol (24:1 v/v) was added, the mixture was agitated on a shaker for 15 min, and centrifuged at 3000 rpm for 5 min. The aqueous top layer was transferred to a new microcentrifuge tube (1.5 ml) containing 400 µl of isopropanol,

Table 1.2. - Susceptible (+) and resistant (-) reaction of common bean differential cultivars to races of *C. lindemuthianum*. Races were previously reported in Brazil, Canada, Colombia, Guatemala, Mexico, the Netherlands, Peru, and the United States and characterized using RAPDs in the present study.

Race[a]	Country[b]	GP[c]	Differential Cultivars[d]												Isolate[f]	Reference
			A	B[e]	C[e]	D	E	F[e]	G	H	I	J	K	L		
2	Peru	A	-	+	-	-	-	-	-	-	-	-	-	-	CL 52 Per[g]	Restrepo, 1994
3	Peru, Neth	A	+	+	-	-	-	-	-	-	-	-	-	-	CL 3 Per[g]	Restrepo, 1994
5*	Peru	A	+	-	+	-	-	-	-	-	-	-	-	-	CL 61 Per[g]	Restrepo, 1994
7	Peru	A	+	+	+	-	-	-	-	-	-	-	-	-	CL 73 Per[g]	Restrepo, 1994
15*	Clb	A	+	+	+	+	-	-	-	-	-	-	-	-	CL 262 Clb[g]	Restrepo, 1994
17	Can, US	M	+	-	-	-	+	-	-	-	-	-	-	-	MSU[h]	Barrus, 1918 Tu et al., 1984
19	Neth	A	+	+	-	-	+	-	-	-	-	-	-	-	MSU[h]	-
23*	Bra,US,Neth	A	+	+	+	-	+	-	-	-	-	-	-	-	ATCC 18789	Andrus and Wade, 1942
31*	Bra, Neth	M	+	+	+	+	+	-	-	-	-	-	-	-	3369-M[i]	Drifhout and Davis, 1989, Menezes and Dianese, 1988
38*	DR	A	-	+	+	-	-	+	-	-	-	-	-	-	CL 11 Dom[g]	-
55	Bra	A	+	+	+	-	+	+	-	-	-	-	-	-	ClCNF 264[j]	Menezes and Dianese, 1988
63	-	-	+	+	+	+	+	+	-	-	-	-	-	-	MSU[h]	Drifhout and Davis, 1989
64	Mex	M	-	-	-	-	-	-	+	-	-	-	-	-	64[k]	Drifhout and Davis, 1989

Table 1.2. (cont'd)

81	Bra, Neth	M	+	-	-	-	+	-	+	-	-	-	-	-	ClCNF 538[j]	Menezes and Dianese, 1988
87*	Bra	M	+	+	+	-	+	-	+	-	-	-	-	-	ClCNF 534[j]	Menezes and Dianese, 1988
102*	Bra, US	A	-	+	+	-	-	+	+	-	-	-	-	-	ClCNF 389[j]	Barrus, M. F. 1918
																Menezes and Dianese, 1988
141	Guat	M	+	-	+	+	-	-	-	+	-	-	-	-	MSU[h]	Schwartz et al., 1982
130*	Can, US	A	-	+	-	-	-	-	-	+	-	-	-	-	MSU[h]	Tu et al., 1984
																Barrus, 1918
192*	Mex	M	-	-	-	-	-	-	+	+	-	-	-	-	192[k]	Drifhout and Davis, 1989
384*	Mex	M	-	-	-	-	-	-	-	+	+	-	-	-	384[k]	Drifhout and Davis, 1989
453	Bra	M	+	-	+	-	+	-	+	+	+	-	-	-	ClCNF 340[j]	Menezes and Dianese, 1988
1024*	Mex	M	-	-	-	-	-	-	-	-	-	-	+	-	1024[k]	Drifhout and Davis, 1989
1473*	Mex	M	+	-	-	-	-	-	+	+	+	-	+	-	CL 91[g]	Pastor-Corrales et al., 1994
2047*	CR	M	+	+	+	+	+	+	+	+	+	+	+	-	CL 38 CR[g]	Pastor-Corrales et al., 1995

[a] Designation of races based on the binary nomenclature system (Pastor-Corrales, 1991). Races previously designated by Greek letters: 17 - alpha (α), 23 - delta (δ), 31 - kappa (κ), 55 - lambda (λ), 81 - eta (ξ), 87 - mu (μ), 102 - gamma (γ), 130 - beta (β), 453 - zeta (ζ) (Balardin and Kelly, 1997)

[b] Countries: Bra (Brazil), Can (Canada), Clb (Colombia), CR (Costa Rica), DR (Dominican Republic), Guat (Guatemala), Mex (Mexico), Neth (Netherlands), and US (United States)

[c] Host Gene Pool: A – Andean; M – Middle American

[d] Common bean differential cultivars used to characterize races of *C. lindemuthianum* followed by their respective binary value (Pastor-Corrales, 1991): A (Michelite - 1), B (Michigan Dark Red Kidney - 2), C (Perry Marrow - 4), D (Cornell 49242 - 8), E (Widusa - 16), F (Kaboon - 32), G (Mexico 222 - 64), H (PI 207262 - 128), I (TO - 256), J (TU - 512), K (AB 136 - 1024), and L (G 2333 - 2048). Resistance genes characterized in cultivars from the common bean differential series (Young and Kelly, 1996; Young

et al., 1997): B (Michigan Dark Red Kidney - *Co-1*), D (Cornell 49242 - *Co-2*), G (Mexico 222 - *Co-3*), I (TO - *Co-4*), J (TU - *Co-5*), K (AB 136 - *Co-6*), and L (G2333 – *Co-4*^{2}, *Co-5*, and *Co-7*

[e] Cultivars belonging to the Andean gene pool

[f] Isolate source (research center and researcher):

[g] CIAT – Centro Internacional de Agricultura Tropical, Cali, Colombia - (M.A. Pastor-Corrales)

[h] Michigan State University, Dry Bean Breeding and Genetics Lab, East Lansing, United States - (J.D. Kelly)

[i] Instituto Agronômico do Paraná, Londrina, Brazil - (J.R. Menezes)

[j] Centro Nacional de Pesquisa de Feijão e Arroz - EMBRABA, Goiânia, Brazil - (C. Rava)

[k] Mexican National Bean Research Program – INIFAP, Mexico - (R. Rodriguez and J. Acosta)

*Isolates analyzed by Fitch-Margoliash cluster analysis in addition to the isolates listed in Table 1.1 (Figure 1.1).

mixed well, and incubated at room temperature for 5 min. The emulsion was centrifuged at 3500 rpm for 5 min, the supernatant was discarded, and tubes were inverted for 5 min to allow complete evaporation of the isopropanol. The pellet was dissolved in 100 µl TE (10 mM tris-HCl, pH 8.0, 0.5 mM EDTA), 10 $\mu g.ml^{-1}$ RNAse A were added, and the emulsion was incubated for 10 min at room temperature. Adding 50 µl cold 100% EtOH precipitated DNA. After 10 min of incubation at room temperature, the mixture was centrifuged at 3500 rpm for 5 min, and the pellet was washed twice with EtOH (100%). Extracted DNA was dissolved in 100 µl TE and stored at -20°C. Concentration of DNA samples was standardized to 10 $ng.\mu l^{-1}$ by DNA fluorometry (TKO 100, Hoefer Scientific, San Francisco CA).

The PCR procedure reported by Williams et al. (1990) was followed with minor modifications. Approximately 1.6 µl of genomic DNA template (10 $ng.\mu l^{-1}$) and 1.6 µl of single decamer primer (10 $ng.\mu l^{-1}$) (Operon Technologies Inc., Alameda CA) was used in a 18.22 µl amplification reaction. The reaction contained also 0.15 µl of Stoffel Fragment Polymerase (10 units/µl) (Perkin Elmer Cetus, Norwalk, CN), 1x Stoffel buffer (10x), 5 mM $MgCl_2$ (25 mM), and 2 mM of dNTPs (200 mM) (Perkin Elmer Cetus), overlaid with 25 µl of sterile mineral oil prior to amplification. To ensure that amplification product was not primer artifacts (Williams et al., 1990), genomic DNA was omitted from a control reaction included for each primer examined.

Amplification was performed in a DNA thermal cycler (Perkin Elmer 480 Cetus) programmed for one initial denaturation cycle (93°C/3 min), 40 step-cycles (94°C/45 sec; 38°C/45 sec; 72°C/1 min), one time-delay cycle (72°C/10 min), and a universal soak cycle (8°C). Amplified RAPD products were electrophoresed at 70 V for 3.5 h on 1.2%

agarose gel using 0.5x TBE (45 mM tris-borate, 1 mM EDTA. pH 7.0) running buffer. RAPD products were observed on 0.005% EtBr-stained agarose gel under UV light after electrophoresis. Ten µl lambda DNA, restricted with *Hind* III and *EcoR* I were included as molecular weight marker.

A total of 311 primers were initially screened to select polymorphic primers for races 3, 9, 17, 65, 73, 89, 102, 130, 201, 384, 449, 453 of *C. lindemuthianum*. These races were chosen because of their pathogenicity to the first nine-anthracnose differential cultivars. Eight primers were selected (OPA-9, GGGTAACGCC; OPF-13, GGCTGCAGAA; OPG-2 GGCACTGAGG; OPG-3, GAGCCCTCCA; OPS-17, TGGGGACCAC; OPT-14, AATGCCGCAG; OPV-7, GAAGCCAGCC; and OPV-10, GGACCTGCTG) which consistently generated major polymorphic amplicons in all isolates. Selected primers were then tested on all 60 isolates of *C. lindemuthianum*. Molecular polymorphism among isolates with identical virulence phenotypes was investigated. Isolates from races 3, 17, 23, 31, 55, 65, 73, 81, 102, 130 and 453 from the same and different countries, and isolates from races 65 and 73 from the same country only, were tested with the eight selected primers. Two replicates of the RAPD assay were run with different template DNA obtained from different DNA isolations.

Data analysis. The virulence and molecular data were analyzed separately using cluster analyses. A single isolate of each unique combination of virulence and molecular phenotypes was included in the analyses.

Separate data matrices were generated for the virulence and molecular data by scoring resistance as 0 and susceptibility as 1, and absence or presence of RAPD

amplicon as 0 or 1, respectively. RAPD amplicons were considered polymorphic if shared by fewer than 57 of the 60 isolates of *C. lindemuthianum*. Similarity matrices for both virulence and molecular data were derived with the SIMQUAL program in the Numerical Taxonomy and Multivariate Analysis System for personal computer (NTSYS-pc) version 1.70 (Exeter Software, Setauket NY). The DICE and Jaccard's coefficients were used to compute distances in virulence and RAPDs data, respectively. The ratio between presence of characters among two individuals and all possible combination of unmatched characters among the same individuals is considered by both coefficients, although DICE weights matches twice that of mismatches. The FITCH program in the Phylogeny Inference Package (PHYLIP) version 3.5c (Department of Genetics, University of Washington, Seattle WA) was used to estimate clustering from the distance matrix data using the Fitch-Margoliash method. Phenograms for both virulence and molecular data were produced using the DRAWGRAM program (PHYLIP).

RESULTS

Virulence phenotypes. Forty-one races were identified among the 138 *C. lindemuthianum* isolates tested for virulence (Table 1.1). Races fell into two categories, those found over a wide geographic area (i.e., different continents) and those restricted to a single country. Only three races (7, 65 and 73) were widely distributed. Race 73 was the most common, comprising 28% of the total sample. This race was found in four separate countries and was isolated repeatedly in North, South and Central America. Race 7 was found at low frequency but extremely widespread, being detected once in South and Central America, but it was isolated repeatedly from only the United States. The third

race, 65, was moderately common (14% of all isolates), but was isolatedly repeated from only Brazil and the United States. The remaining races were detected in only a single country, with about a third of these localized races being isolated multiple times within a country. These races may be locally common, but geographically restricted. Variability was highest in Central America (76%; i.e., number of detected races /number of isolates from a specific region) and decreased either north (North America 7%) or south (South America 17%).

Although 39% (16 of 41) of the races were detected multiple times and three races were widespread, no race was isolated from plants from both *P. vulgaris* gene pools. For example, races 65 and 73 were common and widespread, but they were always isolated from hosts of the Middle American gene pool. In contrast, race 7 was isolated from three different countries but always from host from the Andean gene pool.

Races that were isolated from Middle American hosts were virulent to all cultivars present in the differential series except cultivar L (Table 1.1). These races can be categorized into two groups. One group was virulent to specific resistance genes (*Co-2*, *Co-3*, *Co-4*, *Co-5*, and *Co-6*). The majority of races from this group attacked differentials A and G. The differentials with the *Co-2* and *Co-4* genes were mostly susceptible to races from Honduras and Mexico, respectively. Races 1545, 1600, 1601, 1673, 1929, 1993 were virulent to both the *Co-5* and *Co-6* genes. Races from Honduras and Mexico were the only races virulent to the *Co-6* gene. Races virulent to genotypes belonging to both gene pools formed the second group. Race 357 was virulent to the Andean cultivars C and F and the Middle American cultivars G and I. Races 453, 469, 1165, 1431, 1677 and 1741 attacked the Andean cultivar C. Races 453 and 469 were

virulent to the Middle American cultivars G, H and I. Races 1165, 1431, 1677 and 1741 caused disease on the Middle American cultivar K.

The group of races isolated from Andean hosts was virulent to resistance genes from the Andean and Middle American gene pools. Race 2, 7, and 19 were virulent to the *Co-1* gene in cultivar B, whereas race 7 was also virulent to the resistance genes in the C cultivar. Race 55 was virulent to all three Andean cultivars B, C and F. These Andean races were also virulent to the Middle American cultivars, A and E.

The phenetic analysis included 54 virulence phenotypes of *C. lindemuthianum*; 41 races identified in this survey (Table 1.1), and 13 additional races previously reported from Brazil, Canada, Colombia, the Dominican Republic, Guatemala, Mexico, the Netherlands, Peru and the United States (Table 1.2). The races clustered into three groups, with one of these groups containing a single race, 2047 (Figure 1.1). There were no obvious factors that correlated with the virulence clusters. The two multiple isolate clusters contained isolates from hosts of Andean and Middle American ancestry, and both clusters contained isolates from North, South and Central America. However, isolates from South America were slightly more common in cluster 2. Races that are known to be widespread geographically were also found in clusters 1 and 2.

There were no obvious patterns within clusters 1 and 2, with races obtained from different host gene pools often clustered tightly (e.g., 1033 and 23; 320 and 102; 31 and 2). No geographic patterning was evident within a cluster since isolates from each geographic region were spread throughout each cluster.

Molecular variation. Sixty isolates of *C. lindemuthianum* were separated based on amplification product patterns from 312 combinations of primer-DNA templates. Primers OPA-9, OPF-13, OPG-2, OPG-3, OPS-17, OPT-14, OPV-7, and OPV-10 generated 11, 10, 10, 9, 7, 17, 11, and 18 distinct and reproducible polymorphic amplicons, respectively. Ten RAPD amplicons were monomorphic. Fifteen separate clusters were distinguished using the RAPD data (Figure 1.2). The number of isolates within a cluster ranged from 19 for cluster 2 to one for seven separate clusters. There was no congruence between the RAPD and virulence phenograms. Isolates that were identical for virulence (i.e., the same race) were most often dissimilar for RAPD markers. For example, the four isolates that were classified as race 73 were distributed across three different clusters within the RAPD phenogram. RAPD-patterns obtained with primer OPA-09 and OPG-02 are shown (Figure 1.3, Figure A1.1). The analyses of isolates from race 73 showed polymorphism within and among countries (Figure 1.4). Isolates from race 73 characterized in Honduras and Mexico showed two distinct RAPD patterns among isolates from each country, whereas only two (lanes q and s) of the 13 isolates from the United States were polymorphic (Figure 1.4). Isolates from race 65 characterized in the United States showed different RAPD-pattern, whereas isolates from Brazil were monomorphic. Of the ten races that displayed intra-race variability for RAPD phenotype, only isolates from races 55 and 453 clustered within the same group of the RAPD phenogram. The clustering in the RAPD phenogram was not associated with geography or the host gene pool from which the isolate was obtained. The two largest clusters, 1 and 2, contained isolates from all geographic regions sampled, and were collected on hosts from both *P. vulgaris* gene pools. Even clusters that contained few isolates were

Figure 1.1. Phenogram of *C. lindemuthianum* races based on virulence to *Phaseolus* differential cultivars. DICE (NTSYS-pc) generated a distance matrix data of virulence data that was used in the FITCH program (PHYLIP) to estimate phylogeny. A. Binary identification (Pastor-Corrales, 1991) of *C. lindemuthianum* races. B. Origin of isolates: Arg (Argentina), Bra (Brazil), Clb (Colombia), CR (Costa Rica), DR (Dominican Republic), Hon (Honduras), Per (Peru), Mex (Mexico), and US (United States); Div (races identified from isolates collected in different countries). C. Middle American (M) and Andean (A) race groups according to host source.

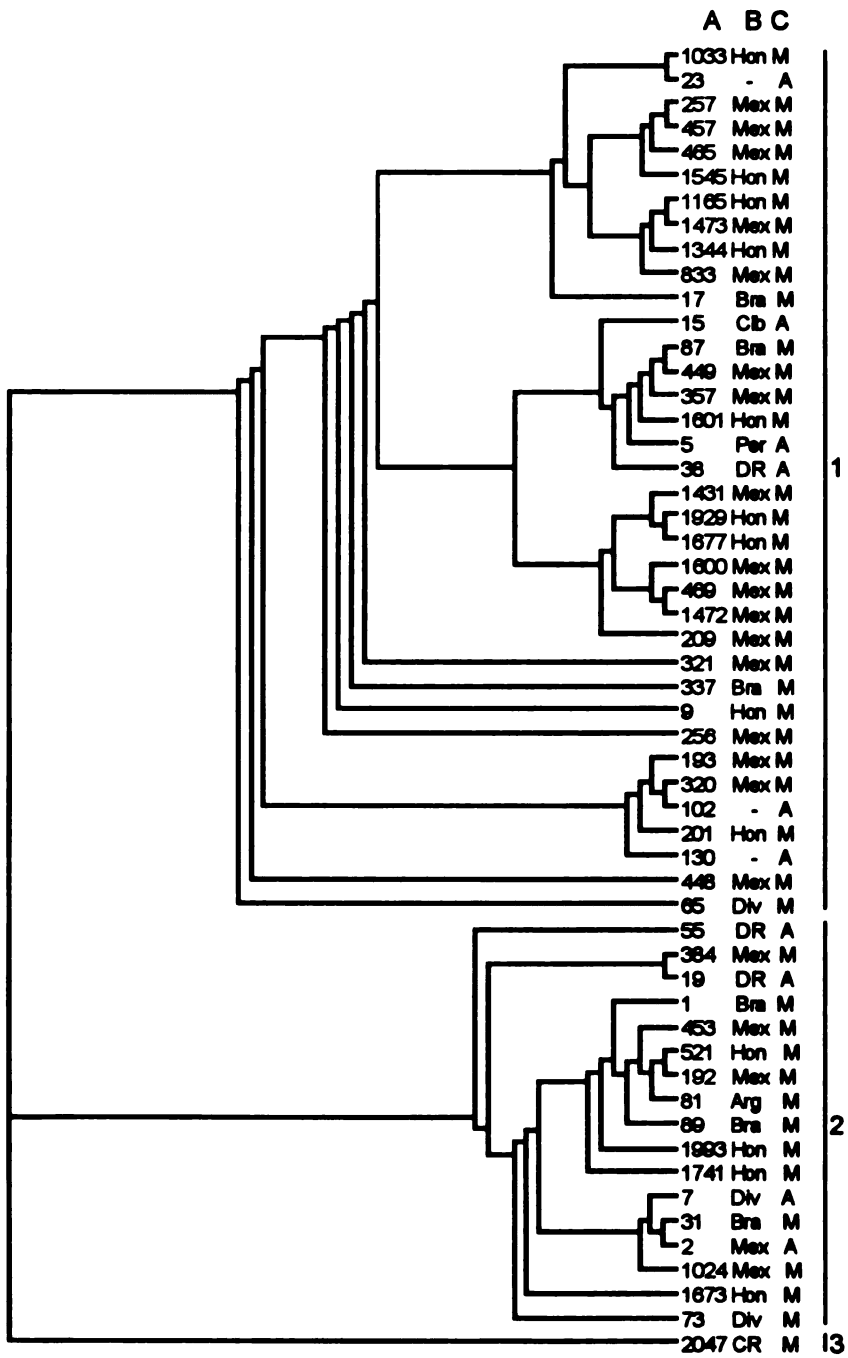
A B C
1033 Hon M
23 - A
257 Mex M
457 Mex M
465 Mex M
1545 Hon M
1165 Hon M
1473 Mex M
1344 Hon M
833 Mex M
17 Bra M
15 Clb A
87 Bra M
449 Mex M
357 Mex M
1601 Hon M
5 Per A
38 DR A
1431 Mex M
1929 Hon M
1677 Hon M
1600 Mex M
469 Mex M
1472 Mex M
209 Mex M
321 Mex M
337 Bra M
9 Hon M
256 Mex M
193 Mex M
320 Mex M
102 - A
201 Hon M
130 - A
448 Mex M
65 Div M
55 DR A
384 Mex M
19 DR A
1 Bra M
453 Mex M
521 Hon M
192 Mex M
81 Arg M
89 Bra M
1993 Hon M
1741 Hon M
7 Div A
31 Bra M
2 Mex A
1024 Mex M
1673 Hon M
73 Div M
2047 CR M
1
2
3

Figure 1.1

Figure 1.2. Phenogram of *C. lindemuthianum* races based on RAPD analysis. Jaccard's (NTSYS-pc) generated a distance matrix data. FITCH program (PHYLIP) version 3.5c estimated phylogeny from the distance matrix data using the Fitch-Margoliash method. A. Binary identification of *C. lindemuthianum* races (Pastor-Corrales, 1991); B. Origin of races: Bra (Brazil), Can (Canada), Clb (Colombia), CR (Costa Rica), DR (Dominican Republic), Gua (Guatemala), Hon (Honduras), Per (Peru), Mex (Mexico), and US (United States); C. Middle American (M) and Andean (A) groups of races according to host source. * Isolate obtained from the Michigan State University / Dry Bean Breeding and Genetics Lab. ** Isolates obtained from Centro Internacional de Agricultura Tropical – Bean Pathology Program.

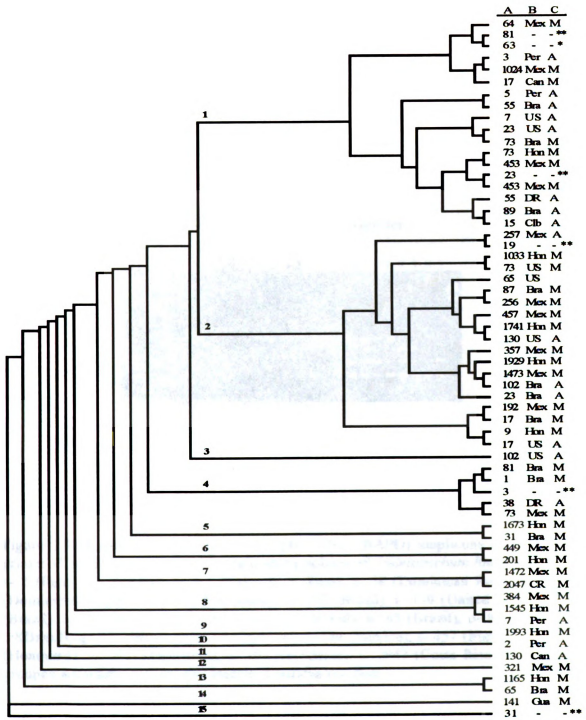
A B C
64 Mex M
81 - - **
63 - - *
3 Per A
1024 Mex M
17 Can M
5 Per A
55 Bra A
7 US A
23 US A
73 Bra M
73 Hon M
453 Mex M
23 - - **
453 Mex M
55 DR A
89 Bra A
15 Clb A
257 Mex A
19 - - **
1033 Hon M
73 US M
65 US
87 Bra M
256 Mex M
457 Mex M
1741 Hon M
130 US A
357 Mex M
1929 Hon M
1473 Mex M
102 Bra A
23 Bra A
192 Mex M
17 Bra M
9 Hon M
17 US A
102 US A
81 Bra M
1 Bra M
3 - - **
38 DR A
73 Mex M
1673 Hon M
31 Bra M
449 Mex M
201 Hon M
1472 Mex M
2047 CR M
384 Mex M
1545 Hon M
7 Per A
1993 Hon M
2 Per A
130 Can A
321 Mex M
1165 Hon M
65 Bra M
141 Gua M
31 - - **
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 1.2

Figure 1.3. Randomly amplified polymorphic DNA (RAPD) amplicons obtained with primer 17 of Operon kit S for 24 single-spore isolates of *Colletotrichum lindemuthianum*: a- 2 (Peru), b- 3 (Peru), c- 5 - (Peru), d- 7 (Peru), e- 38 (Dominican Republic), f- 55 (Dominican Republic), g- 15 (Colombia), h- 102 (Brazil), i- 130 (United States), j- 23 (Brazil), k- 87 (Brazil), l- 31 (Brazil), m- 17 (Brazil), n- 65 (Brazil), o- 81 (Brazil), p- 89(Brazil), q- 453 (Mexico), r- 73 (Mexico), s- 449 (Mexico), t- 457 (Mexico), u- 1673 (Honduras), v- 1993 (Honduras), x- 201 (Honduras), y- 2047 (Costa Rica). Races were grouped according Andean and Middle American reaction.

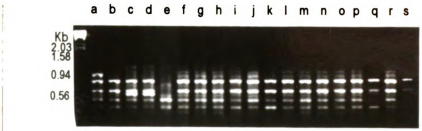

Figure 1.4. Randomly amplified polymorphic DNA (RAPD) patterns obtained with primer 13 of Operon kit F for 19 single-spore isolates from races 73 of *Colletotrichum lindemuthianum* collected in Honduras (lanes a to d), Mexico (lanes e and f), and the United States (lanes g to s).

geographically variable. For example clusters 4, 6, 8 and 13 contained isolates from both Central and South America. Clusters 4 and 8 also contained isolates obtained from hosts of Andean and Middle American ancestry. The only geographic tendency was for isolates from North America to be found predominantly in clusters 1 and 2.

DISCUSSION

Molecular markers are used extensively to characterize plant pathogens (Crowhurst et al., 1991; Guthrie et al., 1992; Michelmore and Hulbert, 1987; Mills et al., 1992). When combined with data on virulence, these markers can often elucidate the population genetic structure and evolutionary relationships of plant pathogens (McDonald and McDermott, 1993; Milgroom, 1995; Sreenivasaprasad et al., 1992). This information can suggest novel strategies for the control of pathogens. For example, rice blast populations, *Pyricularia oryzae*, are often composed of several distinct lineages, with each lineage being virulent on a sub-set of available host cultivars (Casela et al., 1995; Levy et al., 1993). Because of this structure, breeding programs have concentrated on obtaining resistance against specific pathogen lineages and not virulence phenotypes (Casela et al., 1995; Levy et al., 1993).

The division of common beans, *P. vulgaris*, into Andean and Middle American gene pools (Gepts, 1988; Singh et al., 1991), suggests the possibility of a similar lineage based control strategy. If pathogens are specialized on one of the host gene pools, then transferring resistance genes between gene pools may provide a more durable resistance (Gepts, 1988; Singh et al., 1991). Common bean rust (*Uromyces phaseoli*; Maclean et al., 1995; Stavely, 1982; Stavely 1984) and angular leaf spot (*Phaeoisariopsis griseola*;

Guzman et al., 1995) appear to be specialized in congruence with the two *P. vulgaris* gene pools. A previous study of twelve *C. lindemuthianum* isolates found that RAPD and restriction fragment length polymorphism (RFLP) markers divided the species into two groups (Fabre et al., 1995). While not absolute, the groupings suggested some specialization within *C. lindemuthianum* that corresponded to the two gene pools within *P. vulgaris*. Pastor-Corrales (Pastor-Corrales, 1996) reported that *C. lindemuthianum* can be divided into two groups based on virulence; one specializing on hosts from the Middle American gene pool and the second specializing on Andean hosts.

Our data for virulence phenotypes and RAPD markers suggest that *C. lindemuthianum* is not structured in congruence with host gene pools. Virulence phenotypes clustered predominantly into two large groups, but each group contained races isolated from both of the host gene pools. The virulence data can also be examined for the ability of races, isolated from hosts from one gene pool, to infect differential cultivars from the other gene pool. Differential cultivars B, C and F, which represent the Andean gene pool, were susceptible to 3%, 17% and 3%, respectively, of the 37 races isolated from host of Middle American origin (Table 1.1). Further, some of the isolates, previously collected from Middle American hosts, were found to infect these Andean cultivars (Table 1.2). Three of the four *C. lindemuthianum* races obtained from Andean hosts could infect at least one differential cultivar from the Middle American gene pool (Table 1.1). We note, however, that these results are based on greenhouse inoculations. Despite the ability of some races to overcome resistance genes from both the Middle American and Andean host gene pools, each race was actually collected from hosts of only one gene pool.

A previous study reported that widespread races (i.e., races found in five or more Latin American countries) of *C. lindemuthianum* infected a smaller number of differential cultivars (Pastor-Corrales, 1996). Our data tend to agree with this finding; the three widespread races, 7, 65 and 73, could attack two or three differentials, while the average number of differentials infected by a race was 3.73. Although our sampling was not designed to explicitly measure diversity within an area, our data suggest that *C. lindemuthianum* is more variable in Central America, than in either South or North America. This finding is consistent with the earlier work of Pastor-Corrales (Pastor-Corrales, 1996) who found that the *C. lindemuthianum* population in Central America was more diverse than isolates from Andean areas. Also, genetic diversity of *C. lindemuthianum* populations was higher in Mexico compared with Ecuador and Argentina (Sicard et al., 1997).

RAPD markers separated our isolates into 15 clusters. The clustering within the RAPD phenogram was not congruent with either geographic location or host gene pool; nor was there any congruence with the virulence phenogram. Thus, the RAPD primers utilized here cannot be used for grouping *C. lindemuthianum* according to the host gene pool. It is not clear to what degree our RAPD phenogram represents phylogentic relationships within *C. lindemuthianum*. Recent reports have pointed out a number of problems associated with assessing phylogenetic relationships using RAPD markers (Palumbi, 1996). Our groupings differ from an earlier report based on twelve *C. lindemuthinaum* isolates (Fabre et al., 1995), which found two groups using both RAPD markers and two different sets of RFLP markers from the ribosomal genes. Sicard et al. (1997) grouped *C. lindemuthianum* isolates using RFLP of the amplified ribosomal

internal transcribed spacer region (rDNA-ITS). The authors demonstrated that the three groups corresponded to the host gene pools. These results suggest an adaptation of strains on cultivars of the same geographic origin.

Taken together, our data indicate that *C. lindemuthianum* may not be highly structured to specific *P. vulgaris* gene pools. Therefore, control strategies that transfer resistance genes from one *P. vulgaris* gene pool to the other may not confer durable resistance to *C. lindemuthianum*. We urge caution in the utilization of this strategy until races are further evaluated, under field conditions, for their ability to infect hosts from both gene pools, and a more comprehensive analysis of the evolutionary relationships within *C. lindemuthianum* is completed.

LIST OF REFERENCES

Andrus, C. F., and Wade, B.L. 1942. The factorial interpretation of anthracnose resistance in beans. Pages 1-29 in: Tech. Bull. No. 310. USDA. Washington.

Balardin, R., and Kelly, J.D. 1996. Identification of Race 65-epsilon of bean anthracnose (*Colletotrichum lindemuthianum*) in Michigan. Plant Dis. 80:712.

Balardin, R. S., Pastor-Corrales, M.A., and Otoya, M.M. 1990. Variabilidade patogênica de *Colletotrichum lindemuthianum* no Estado de Santa Catarina. Fitopatol. Bras. 15:243-245.

Balardin, R. S. and Kelly, J. D. 1997. Re-characterization of *Colletotrichum lindemuthianum* races. Ann. Rep. Bean Improv. Coop. 40:126-127.

Barrus, M. F. 1918. Varietal susceptibility of beans to strains of *Colletotrichum lindemuthianum* (Sacc. & Magn.) B. & C.. Phytopathology 8:589-605.

Blondet, A. 1963. L'anthracnose du haricot: etudé des races physiologiques du *Colletotrichum lindemuthianum*. Ph.D. thèse, Faculté de Sciense, Paris, France.

Burkholder, W. H. 1923. The gamma strain of *Colletotrichum lindemuthianum* (Sacc. et Magn.) B.et C.. Phytopathology 13:316-323.

Casela, C. R., Ferreira, A.S., Zeller, K.A., Levy, M. 1995. Pathotype variation in the sorghum anthracnose fungus: a phylogenetic perspective for resistance breeding. Pages 257-288 in: Disease analysis through genetics and biotechnology. J. F. Leslie and R. S. Frederiksen, eds. Iowa State University Press. Ames, IO.

Crowhurst, R. N., Hawthorne, B.T., Rikkerink, E.H.A., Templeton, M.D. 1991. Differentiation of *Fusarium solani* f.sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Curr. Genet. 20:391-396.

Drifhout, E., and Davis, J.H.C. 1989. Selection of a new set of homogeneously reacting bean (*Phaseolus vulgaris*) differentials to differentiate races of *Colletotrichum lindemuthianum*. Plant Pathology 38:391-396.

Duvick, D.N. 1996. Plant Breeding, an evolutionary concept. Crop Sci. 36:539-548.

Edwards, K. C., Johnstone, C., and Thompson, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19:1349.

Fabre, J. V., Julien, J., Parisot, D., and Dron, M. 1995. Analysis of diverse isolates of *Colletotrichum lindemuthianum* infecting common bean using molecular markers. Mycol. Res. 99:429-435.

Fouilloux, G. 1979. New races of bean anthracnose and consequences on our breeding programs. Pages 221-235 in: Disease of tropical food crops. H. Maraite and J.A. Meyer, eds. Louvain-la-Neuve, Belgium.

Garrido, E. R. 1986. Identificación de razas fisiológicas de *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scrib. en Mexico y busqueda de resistência genética a este hongo. MSc thesis, Institución de Ensenanza y Investigación en Ciencias Agrícolas, Montecillos, Mexico.

Gepts, P. 1988. A Middle American and an Andean common bean gene pool. Pages 375-390 in: Genetic resources of *Phaseolus* beans; their maintenance, domestication, and utilization. P. Gepts, ed. Kluwer. London.

Guthrie, P. A. I., Magill, C.W., Frederiksen, R.A., and Odvody, G.N. 1992. Random amplified polymorphic DNA markers: a system for identifying and differentiating isolates of *Colletotrichum graminicola*. Phytopathology 82:832-835.

Guzman, P., Gilbertson, R.L., Nodari, R., Johnson, W.C., Temple, S.R., Mandala, D., Mkandawire, A.B.C., and Gepts, P. 1995. Characterization of variability in the fungus *Phaeoisariopsis griseola* suggests coevolution with the common bean (*Phaseolus vulgaris*). Phytopathology 85:600-607.

Kelly, J. D., Afanador, L., and Cameron, L.S. 1994. New races of *Colletotrichum lindemuthianum* in Michigan and implications in dry bean resistance breeding. Plant Dis. 78:892-894.

Kruger, J., Hoffmann, G.M., and Hubbeling, N. 1977. The kappa race of *Colletotrichum lindemuthianum* and sources of resistance to anthracnose in *Phaseolus* beans. Euphytica 26:23-25.

Levy, M., Correa-Victoria, J., Zeigler, R.S., Xu, S., and Hamer, J. 1993. Genetic diversity of the rice blast fungus in a disease nursery in Colombia. Phytopathology 83:1427-1433.

Maclean, D. J., Braithwaite, K.S., Irwin, J.A.G., Manners, J.M., and Groth, J.V. 1995. Random Amplified Polymorphic DNA reveals relationships among diverse genotypes in Australian and American collections of *Uromyces appendiculatus*. Phytopathology 85:757-765.

McDonald, B. A., and McDermott, J. M. 1993. Population genetics of plant pathogenic fungi. BioScience 43:311-319.

Menezes, J. R., and Dianese, J.C. 1988. Race characterization of Brasilian isolates of *Colletotrichum lindemuthianum* and detection of resistance to anthracnose in *Phaseolus vulgaris*. Phytopathology 78:650-655.

Michelmore, R. W., and Hulbert, S.H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol. 25:383-404.

Milgroom, M. G. 1995. Analysis of population structure in fungal plant pathogens. Pages 213-229 in: Disease analysis through genetics and Biotechnology. J. F. Leslie, and R.A. Frederiksen, eds. Iowa State University Press. Ames, IO.

Mills, P. R., Sreenivasaprasad, S., and Brown, A.E. 1992. Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. FEMS Microbiology Letters 98:137-144.

Palumbi, S.R. 1996. Nucleic acids II: the polymerase chain reaction. Pages 205-247 in: Molecular Systematics. D.M. Hills, C. Moritz, and B.K. Mable, eds. Sinauer, Sunderland, MA.

Pastor-Corrales, M. A. 1991. Estandarización de variedades diferenciales y de designación de razas de *Colletotrichum lindemuthianum*. Phytopathology 81:694 (abstract).

Pastor-Corrales, M. A. 1996. Traditional and molecular confirmation of the coevolution of beans and pathogens in Latin America. Ann. Rep. Bean Improv. Coop. 39:46-47.

Pastor-Corrales, M. A., Erazo, O.A., Estrada, E. I., and Singh, S. P. 1994. Inheritance of anthracnose resistance in common bean accession G 2333. Plant Dis. 78:959-962.

Pastor-Corrales, M. A., Otoya, M.M., and Molina, A. 1995. Resistance to *Colletotrichum lindemuthianum* isolates from Middle America and Andean South America in different common bean races. Plant Dis. 79:63-67.

Pastor-Corrales, M. A., and Tu, J.C. 1989. Anthracnose. Pages 77-104 in: Bean production problems in the tropics. H. F. Schwartz, and Pastor-Corrales, M.A., eds. CIAT. Cali, Colombia.

Rava, C. A., Molina, J., Kauffmann, M., and Briones, I. 1993. Determinación de razas fisiológicas de *Colletotrichum lindemuthianum* en Nicaragua. Fitopatol. Bras. 18:388-391.

Restrepo, S. 1994. DNA polymorphism and virulence variation of *Colletotrichum lindemuthianum* in Colombia. M.Sc. thesis. Universite Paris IV, Paris-Grignon, France.

Rodriguez-Guerra, R. 1991. Identificación de razas patogenicas de *Colletotrichum lindemuthianum* (Sacc. y Magn.) Scrib. en el estado de Durango mediante un sistema propuesto internacionalmente y respuesta de genótipos de frijol tolerantes a sequia a razas del patógeno. M.Sc., Parasitologia Agricola, Universidad Autonoma Agraria "Antonio Narro", Buenavista, Mexico.

Schwartz, H. F., Pastor-Corrales, M.A., and Singh, S. 1982. New sources of resistance to anthracnose and angular leaf spot of beans (*Phaseolus vulgaris*). Euphytica 31:741-754.

Sicard, D., Michalakis, Y., Dron, M., and Neema, C. 1997. Genetic diversity and pathogenic variation of *Colletotrichum lindemuthianum* in the three centers of diversity of its host, *Phaseolus vulgaris*. Phytopathology, 87:807-813.

Singh, S. P., Gepts, P., and Debouck, D.G. 1991. Races of common bean (*Phaseolus vulgaris*, Fabaceae). Econ. Bot. 45:379-396.

Sreenivasaprasad, S., Brown, A.E., and Mills, P.R. 1992. DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. Physiol. and Mol. Plant Pathol. 41:265-281.

Stavely, J.R. 1982. The potential for controlling bean rust by host resistance. Ann. Rep. Bean Improv. Coop. 25:28-30.

Stavely, J.R. 1984. Pathogenic specialization in *Uromyces phaseoli* in the United States and rust resistance in beans. Plant Dis. 68:95-99.

Tu, J. C., Sheppard, J.W., and Laidlaw, D.M. 1984. Occurrence and characterization of the epsilon race of bean anthracnose in Ontario. Plant Dis. 68:69-70.

Waterhouse, W. L. 1955. Studies of bean anthracnose in Australia. Proc. Linn. Soc. N.S.W. 80:71-83.

Williams, J. G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531-6535.

Young, R. A., and Kelly, J.D. 1996. Characterization of the genetic resistance to *Colletotrichum lindemuthianum* in common bean differential cultivars. Plant Dis. 80:650-654.

Young, R.A., and Kelly, J.D. 1997. RAPD markers linked to three major anthracnose resistance genes in common bean. Crop Sci. 37:940-946.

Young, R.A., Melotto, M., Nodari, R.O., and Kelly, J.D. 1997. Marker assisted dissection of oligogenic anthracnose resistance in the common bean cultivar, G2333. Theor. Appl. Genet. (in press).

Zaumeyer, W.J. and Thomas, H.R. 1957. A monographic study of bean diseases and methods for their control. USDA.

Chapter 2

RIBOSOMAL DNA POLYMORPHISM IN *COLLETOTRICHUM LINDEMUTHIANUM*

ABSTRACT

Intra-specific divergence among isolates of *Colletotrichum lindemuthianum* collected in Argentina, Brazil, Colombia, the Dominican Republic, Honduras, Mexico, Peru and the United States was determined using RFLP analysis and sequencing of the rDNA region of the two internal transcribed spacers (ITS 1 and ITS 2) and the 5.8S rRNA gene. A reproducible 0.58kb fragment was PCR amplified in the 57 isolates including *C. lagenarium* used as an outgroup species. Races were grouped into two clusters by RFLP-ITS analysis. Group I was formed predominantly by Andean races except race 23, which was placed within group II. The Middle American races were observed in both groups. A bootstrap value of 100% in the parsimony and 88% in the neighbor-joining analyses supported a monophyletic group formed by all isolates except race 31. Genetic distances among races of *C. lindemuthianum* ranged from 0.2% to 2.9%. Sequence homology analysis did not show a pattern parallel to a specific host reaction group or associated with an obvious geographic distribution. Likewise, phenetic and parsimony analysis did not show polymorphism in the rDNA region linked to any specific factor. Molecular polymorphism among isolates of races 7, 17, 31, and 73 collected in different countries was demonstrated by RFLP-ITS analysis. Sequence

homology of ITS regions of isolates of race 73 from Mexico and the United States showed the greatest genetic distance value among all isolates. These findings support a level of molecular variability within *C. lindemuthianum* greater than the variability previously characterized by virulence analysis.

INTRODUCTION

Virulence and molecular techniques have been used to characterize variability in *C. lindemuthianum* (Fabre et al., 1995; Sicard et al., 1997, Balardin et al., 1997). Genetic diversity inferred from virulence data is restricted to the virulence genes and does not reflect the whole genetic diversity present in the *C. lindemuthianum* genome (Balardin et al., 1997). The genes involved in host-specificity represent a very small fraction of the genes in the pathogen and may be subjected to strong selective pressure by the host (Leung et al., 1993). Genetic markers that are selectively neutral, reproducible, and relatively easy to assay have been suggested for population genetics analysis (Milgroom, 1995; McDonald, 1997). In addition, the analysis of high numbers of independent loci with no effective selection pressure might give more accurate information about genetic relationships among individuals within a population (Leung et al., 1993). Because a large number of amplicons can be screened in a relatively short period of time, RAPDs are especially useful in differentiating clonal lineages for fungi that reproduce asexually (McDonald, 1997). However, sample purity, lack of reproducibility of PCR reactions among labs, and data interpretation are limitations that might bias the potential role of RAPD markers in phylogenetic analysis (Smith et al., 1995).

Nucleotide sequences offer extremely high resolution of intraspecific diversity and generate data that can be used to estimate sequence divergence (Dowling et al., 1996). The nuclear rDNA sequences coding for the small subunit (18S) and large unit (28S) RNAs show little evolutionary change and sequence data can be used to measure evolutionary divergence among organisms within a population (Hillis et al., 1996). Analyses of closely related groups based on the variable regions within the rRNA genes

have been used to resolve taxonomy ambiguities (Waalwijk et al. 1996; Arora et al., 1996). PCR amplification of ITS region of rDNA was used to characterize variability in *C. gloeosporioides* (Mills et al., 1992). Molecular variability within and between isolates of *C. acutatum*, *C. gloeosporioides* and *C. fragariae* was assessed using rDNA-ITS 1 region (Sreenivasaprasad et al., 1992). Isolates of *C. fragariae* were separated into two groups by homology analyses of ITS sequenced regions, whereas isolates from *C. gloesporioides* were monomorphic. Similarity among isolates of *C. gloesporioides, C. musae, C. fructigenum,* and *C. acutatum* was observed based on the sequence of ITS1 region of *Colletotrichum* spp (Sreenivasaprasad et al., 1994). Sherriff et al. (1994) studied the relationships within the genus *Colletotrichum* using the PCR amplification of rDNA. Their results clustered *C. lindemuthianum, C. malvarum, C. trifolii,* and *C. orbicularae*, whereas *C. gloesporioide* represented a group-species within *Colletotrichum* spp.

Restriction fragment length polymorphism of both nuclear ribosomal genes and the internal transcribed spacer region of the ribosomal genes showed that *C. lindemuthianum* isolates consistently formed two main groups, but some isolates did not fall distinctly into one or the other group (Fabre et al. 1995). The limited number of isolates used in this study did not allow determination of whether the groups were aligned according to host origin or virulence. Using RFLP-ITS, Sicard et al. (1997) grouped isolates of *C. lindemuthianum* collected from wild *Phaseolus* in Mexico, Ecuador and Argentina. Adaptation of races on cultivars of the same geographic origin was suggested because groups corresponded to three host gene pools. In this work we sought to resolve the genetic variation between 45 isolates of *C. lindemuthianum* collected in South, Central and North American countries. Variability within virulence phenotypes was

observed and the effect of host gene pool on *C. lindemuthianum* diversity is discussed.

MATERIALS AND METHODS

Colletotrichum lindemuthianum *isolates, DNA extraction and amplification protocol.* Fifty-seven isolates from Argentina, Brazil, Colombia, Costa Rica, the Dominican Republic, Honduras, Mexico, Peru and the United States were characterized based on RFLP analysis or sequencing of the rDNA region comprising the two internal transcribed spacers (ITS 1 and ITS 2) and the 5.8S rRNA gene (Table 2.1).

DNA was extracted from races of *C. lindemuthianum* according to protocol described in Balardin et al. (1997). PCR amplification of the rDNA region between 3' end of the 18S gene and the 28S gene was conducted using the primers PN3: 5'-CCGTTGGTGAACCAGCGGAGGGATC- 3', and PN10: 5'-TCCGCTTATTGATATGCTTAAG- 3' (Fabre et al. 1995). Approximately 3.0 µl of genomic DNA template (10 ng.μl^{-1}) and 1.5 µl of each primer (10 ng.μl^{-1}) (Biotechnologies Inc., Woodlands, TX) was used in a 30µl amplification reaction. The reaction contained 0.15 µl of Stoffel Fragment Polymerase (10 units/µl) (Perkin Elmer Cetus, Norwalk, CN), 1x Stoffel buffer, 5 mM $MgCl_2$, and 2 mM of dNTPs (Perkin Elmer Cetus), overlaid with 25 µl of sterile mineral oil prior to amplification. Amplification was performed in a DNA thermal cycler (Perkin Elmer 480 Cetus) with the following profile: one initial denaturation cycle (94°C/4 min), 30 step- cycles (94°C/1 min; 55°C/45 sec; 72°C/2 min), one time-delay cycle (72°C/7 min), and a universal soak cycle (8°C).

Table 2.1. Susceptible (+) and resistant (-) reaction of common bean differential cultivars to 57 isolates of *C. lindemuthianum*. Isolates were collected in Argentina, Brazil, Canada, Colombia, Costa Rica, the Dominican Republic, Honduras, Mexico, Netherlands, Peru, and the United States, and characterized using RFLP-ITS analysis

Race[a]	Country	Isolate[b]	GP[f]	Differential Cultivars[g]												RFLP/ITS[h]
				A	B	C	D	E	F	G	H	I	J	K	L	
1	Brazil	Cl. Bra 15.1[c]	M	+	-	-	-	-	-	-	-	-	-	-	-	I[i]
2	Peru	CL 7 Per[d]	A	-	-	+	-	-	-	-	-	-	-	-	-	I
3	Peru	CL 3 Per[d]	A	+	-	+	-	-	-	-	-	-	-	-	-	I
5	Peru	CL 61 Per[d]	A	+	-	-	+	-	-	-	-	-	-	-	-	I
7	Argentina	Cl. Arg 2.1[c]	A	+	+	+	-	-	-	-	-	-	-	-	-	II
	Colombia	CL 187 COL[d]	A	+	+	+	-	-	-	-	-	-	-	-	-	II
	Mexico	Cl Mex 46.1[c]	A	+	+	+	-	-	-	-	-	-	-	-	-	II
	Peru	CL 73 PER[d]	A	+	+	+	-	-	-	-	-	-	-	-	-	II
	United States	Cl. US 12.1[c]	A	+	+	+	-	-	-	-	-	-	-	-	-	I
8	Peru	CL 4 Per[d]	A	-	-	-	-	+	-	-	-	-	-	-	-	II
9	Honduras	Cl. Hon 19.1[c]	M	+	-	-	-	+	-	-	-	-	-	-	-	II
15	Colombia	CL 262 Clb[d]	A	+	-	+	+	+	-	-	-	-	-	-	-	I

Table 2.1 (cont'd)

17	Brazil	Cl. Bra 8.1[c]	M	+	-	-	-	-	+	-	-	-	-	-	-	I
	Canada	MSU[c]	M	+	-	-	-	-	+	-	-	-	-	-	-	II
	United States	MSU[c]	M	+	-	-	-	-	+	-	-	-	-	-	-	I
19	Dominican Republic	Cl. DR 2.1[c]	A	+	-	+	-	-	+	-	-	-	-	-	-	I
23	Brazil	ClCNF 79[e]	A	+	-	+	+	-	+	-	-	-	-	-	-	I
	United States	MSU[c]	A	+	-	+	+	-	+	-	-	-	-	-	-	I
31	Brazil	ClCNF 391[e]	M	+	-	+	+	+	+	-	-	-	-	-	-	II
	Netherlands	CL 13 Eur[d]	M	+	-	+	+	+	+	-	-	-	-	-	-	I
38	Dominican Republic	CL 11 Dom[d]	A	-	-	+	+	-	-	+	-	-	-	-	-	I
39	Dominican Republic	CL 4 DR[d]	A	+	-	+	+	-	-	+	-	-	-	-	-	I
47	Dominican Republic	CL 12 DR[d]	A	+	-	+	+	+	-	+	-	-	-	-	-	I
55	Brazil	ClCNF 264[e]	A	+	-	+	+	-	+	+	-	-	-	-	-	I
65	Brazil	ClCNF 465[e]	M	-	-	-	-	-	-	-	+	-	-	-	-	I
	United States	Cl. US 13.1[c]	M	-	-	-	-	-	-	-	+	-	-	-	-	I
73	Brazil	Cl. Bra 2.1[c]	M	+	-	-	-	+	-	-	+	-	-	-	-	I
	Honduras	Cl. Hon 4.1[c]	M	+	-	-	-	+	-	-	+	-	-	-	-	I
	Mexico	Cl. Mex 24.1[c]	M	+	-	-	-	+	-	-	+	-	-	-	-	II
	United States	Cl. US 6.1[c]	M	+	-	-	-	+	-	-	+	-	-	-	-	I
81	Argentina	Cl. Arg 1.1[c]	M	+		-	-	-	+		+					II
87	Brazil	ClCNF 534[e]	M	+		+	+	-	+		+					II

89	Brazil	ClCNF 265[e]	M	+	-	-	-	+	+	-	+	-	-	-	-	II
102	Brazil	ClCNF 389[e]	A	-	-	+	+	-	-	+	+	-	-	-	-	I
130	Netherlands	MSU[c]	A	-	-	+	-	-	-	-	-	+	-	-	-	II
	United States	MSU[c]	A	-	-	+	-	-	-	-	-	+	-	-	-	II
201	Honduras	Cl. Hon 2.1[c]	M	+	-	-	-	+	-	-	+	+	-	-	-	II
256	Mexico	Cl. Mex 30.1[c]	M	-	-	-	-	-	-	-	-	-	+	-	-	II
257	Mexico	Cl. Mex 10.1[c]	M	+	-	-	-	-	-	-	-	-	+	-	-	II
321	Mexico	Cl. Mex 7.1[c]	M	+	-	-	-	-	-	-	+	-	+	-	-	II
357	Mexico	Cl. Mex 25.1[c]	M	+	-	-	-	-	+	-	+	-	+	-	-	II
384	Mexico	Cl. Mex 34.1[c]	M	+	-	-	+	-	-	+	+	-	+	-	-	II
449	Mexico	Cl. Mex 19.1[c]	M	+	-	-	-	-	-	-	+	+	+	-	-	II
453	Brazil	ClCNF 340[e]	M	+	-	-	+	-	+	-	+	+	+	-	-	II
457	Mexico	Cl. Mex 17.1[c]	M	+	-	-	-	+	-	-	+	+	+	-	-	I
521	Honduras	Cl. Hon 16.1[c]	M	+	-	-	-	+	-	-	-	-	-	+	-	I
833	Mexico	Cl. Mex 13.1[c]	M	+	-	-	-	-	-	-	+	-	+	+	-	II
1033	Honduras	Cl. Hon 15.1[c]	M	+	-	-	-	+	-	-	-	-	-	-	+	I
1165	Honduras	Cl. Hon 10.1[c]	M	+	-	-	+	+	-	-	-	+	-	-	+	I
1472	Mexico	Cl. Mex 9.1[c]	M	-	-	-	-	-	-	-	+	+	+	-	+	II
1545	Honduras	Cl. Hon 12.2[c]	M	+	-	-	-	+	-	-	-	-	-	+	+	I
1601	Honduras	Cl. Hon 22.1[c]	M	+	-	-	-	-	-	-	+	-	-	+	+	I

1673	**Honduras**	**Cl. Hon 1.1[c]**	**M**	+	-	-	+	+	-	-	+	+	-	+	+		**I**
1741	**Honduras**	**Cl. Hon 6.1[c]**	**M**	+	-	-	+	+	-	-	+	+	-	+	+		**I**
1929	**Honduras**	**Cl. Hon 18.1[c]**	**M**	+	-	-	-	+	-	-	-	+	+	+	+		**I**
1993	**Honduras**	**Cl. Hon 20.1[c]**	**M**	+	-	-	-	+	-	-	+	+	+	+	+		**I**
2047	**Costa Rica**	**CL CR 38[d]**	**M**	+	-	+	+	+	+	+	+	+	+	+	+		**I**

[a] Designation of races based on the binary nomenclature system (Pastor-Corrales, 1991). Races previously designated by Greek letters: 17 - alpha (α), 19 – epsilon-Kenya (εK), 23 - delta (δ), 31 - kappa (κ), 55 – lambda (λ), 65 – epsilon (ε), 81 – eta (ξ), 87 – mu (μ), 89 – alpha-Brazil (αB), 102 - gamma (γ), 130 - beta (β), 453 - zeta (ζ) (Balardin and Kelly, 1997).

[b] Isolate source.

[c] Michigan State University, Crop and Soil Sciences, East Lansing, the United States, (J. D. Kelly).

[d] CIAT - International Center for Tropical Agriculture, Cali, Colombia, (M. A. Pastor-Corrales).

[e] Centro Nacional de Pesquisa de Feijão e Arroz - EMBRABA, Goiania, Brazil, (C. Rava).

[f] Host Gene Pool: A – Andean, M – Middle American.

[g] Common bean differential cultivars used to characterize races of *C. lindemuthianum* followed by their respective binary value (Pastor-Corrales, 1991): A (Michelite - 1), B (Michigan Dark Red Kidney - 2), C (Perry Marrow - 4), D (Cornell 49242 - 8), E (Widusa - 16), F (Kaboon - 32), G (Mexico 222 - 64), H (PI 207262 - 128), I (TO - 256), J (TU - 512), K (AB 136 - 1024), and L (G 2333 - 2048).

[h] RFLP of the fragment including ITS1, 5.8S rRNA gene, ITS2.

[i] Clusters I and II revealed by RFLP analysis of the ITS fragment.

Restriction endonuclease digestion of PCR-ITS. The ITS-PCR products were digested with 1 U of restriction endonuclease (*Hae* III, *Msp* I - GibcoBRL Grand Island, NY; *EcoR* I, *Hind* III and *BamH* I - Boehringer Mannheim, Germany) according to the manufacturer's specifications. The digestion reaction contained 10 µl of the amplified product, 2 µl of the digestion enzyme buffer and 8 µl of ddH_2O. The digestion products were electrophoresed at 70 V for 3.5 h on 2% agarose gel using 0.5x TBE (45 mM tris-borate, 1 mM EDTA, pH 7.0) running buffer. RFLP products were observed on 0.005% EtBr-stained agarose gel under UV light after electrophoresis. Ten µl of the 1.5 kb DNA molecular weight marker were included. The experiments were performed twice.

Automated DNA Sequencing. In order to sequence ITS 1 and 2 regions in both directions, PN3 and PN10 were added to individual reaction mixtures after PCR-amplification. For each DNA strand a 20-µl reaction mixture was prepared with 10 µl of the PCR product, 1 µl of each primer, and 9 µl of sterile H_2O. Sequences were obtained using the fluorescent dye dideoxy nucleoside triphosphate terminator method (MSU-DNA Sequencing Facility, East Lansing, MI). Sequencing reactions were run on a polyacrylamide gel using the ABI 373A DNA Sequencer.

Data analysis. Nucleotide sequence data were analyzed using different phylogenetic methods. Sequences of nucleotides were aligned using a subroutine in the SEQUENCHER program based on the CLUSTAL V Method with weighted residues. The nucleotide sequences (Z32986 - ITS1, Sreenivasaprasad et al., 1996; no. Z18975 - ITS2, Sherriff et al., 1994), available in the EMBL database, were used for the sequence

alignment. In order to find the most likely tree topology, phylogenetic trees were generated by both distance and parsimony methods.

The distances among isolates of *C. lindemuthianum* were estimated by the Jukes-Cantor one-parameter method (Jukes and Cantor, 1969) in the DNADIST program, Phylogeny Inference Package (PHYLIP) version 3.5c (Department of Genetics, University of Washington, Seattle WA). The Jukes-Cantor method (J-C) assumes an equal rate of substitution between all pairs of bases as the distance measures the variability. Phylogenetic analysis was carried out by the Neighbor-Joining (N-J) method (Saitou and Nei, 1987) in the NEIGHBOR program (PHYLIP), and Fitch-Margoliash method in the FITCH program (PHYLIP), using the distance matrix computed by J-C method. The N-J method follows the minimum-evolution principle, which consists of choosing the tree with the smallest sum of branch lengths. The Fitch-Margoliash method was used to estimate clustering from the distance matrix computed by J-C method.

The bootstrap was performed by SEQBOOT program (PHYLIP) to determine the confidence limits for the internal branches in each phenogram. An unrooted parsimony analysis was carried out using the DNAPARS program (PHYLIP), which analyzed each bootstrap replicate. Using the CONSENSUS program (PHYLIP) a majority rule consensus tree was generated. The consensus tree consists of monophyletic groups that occur as often as possible in the data, and can be considered an overall estimate of the phylogeny (Felsenstein, 1985).

The patterns of DNA bands originating from RFLP patterns within ITS fragments were compiled in a matrix considering presence (1) and absence (0) of bands. Only the polymorphic patterns inferred from endonuclease digestions were included in the matrix.

The relationships among isolates of *C. lindemuthianum* were estimated using the DOLLO program (PHYLIP). Phenograms were generated by DRAWGRAM program (PHYLIP).

RESULTS

Amplification and RFLP of ITS1, 5.8S, and ITS2 regions. PCR amplification of the region between 18S and 28S genes (ITS1, 5.8S rRNA gene and ITS2) with PN3 and PN10 primers produced a single double-stranded reproducible 0.58 kb fragment in all 57 isolates of *C. lindemuthianum* (Table 2.1) including *C. lagenarium* (Figure 2.1). The endonucleases *Hae* III, *Msp* I, *EcoR* I, *Hind* III and *BamH* I were used to digest the amplified rDNA region of *C. lindemuthianum* isolates. No polymorphisms were observed with *EcoR* I, *Hind* III and *BamH* I although these endonucleases showed restriction sites within ITS 1 and ITS 2 regions. The isolates of *C. lindemuthianum* formed two groups according to restriction of the ITS-DNA region with the endonucleases *Msp* I and *Hae* III (Table 2.1). Group I was formed by isolates exhibiting both the Middle American and Andean reaction, whereas the group II was formed predominantly by isolates with Middle American reaction, except for the presence of the Andean race 23. The geographic origin was not a factor influencing the clustering of isolates of *C. lindemuthinaum*, except the Andean isolates from the Dominican Republic, which were placed within group I (Table 2.1).

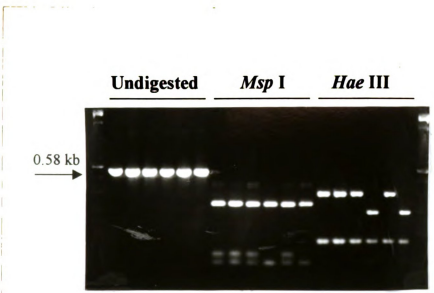

Figure 2.1. PCR amplification products of the ITS1, 5.8S rDNA, and ITS2 region of the rDNA of selected isolates of *C. lindemuthianum*. Lanes 2 to 7 show non-digested ITS fragments of races 7, 8, 31, 65, 1993, and the outgroup species *C. lagenarium*; Lanes 7 to 12 show the same races digested with the restriction enzyme *Msp* I; Lanes 14 to 19 show the same races digested with the restriction enzyme *Hae* III. Lanes 1 and 20 contain the 1.5kb DNA molecular weight marker.

Intra-race variability based on RFLP and sequencing of the ITS1, 5.8S, and ITS2 regions. RFLP analyses of several isolates of races 7, 17, 23, 31, 65, 73, and 130 collected in different countries was carried out to investigate intra-race polymorphism within the ITS region. Of the seven races analyzed, races 23, 65 and 130 did not display intra-race variability (Figure 2.2). Isolates of race 7 characterized in Argentina, Colombia, and Mexico showed distinct RFLP patterns compared to the isolate from the United States. Isolates of race 17 characterized in Canada were different from the isolates collected in Brazil and the United States. Distinct RFLP patterns for isolates of race 31 collected in Brazil and Netherlands was observed. Isolates of race 73 collected in Brazil, Honduras and the United States showed different RFLP banding pattern to the isolate from Mexico. The genetic distances computed by the J-C method based on analysis of sequences of *C. lindemuthianum* showed that race 73 from Mexico had the greatest genetic distance from isolates of races CR 2047, US 23 and US 73 (Table 2.2). The genetic dissimilarity among isolates of race 73 was supported by results from RFLP-ITS. Genetic similarity among races was observed despite the host reaction group or geographic origin of races. For instance, the shortest genetic distance was observed among the Middle American races 8, 81, 89, 457 and 2047 and the Andean races 5, 15, and 23 (Table 2.2). Races 8 and 5 were collected in Peru and the other races were collected in Argentina, Brazil, Colombia, Costa Rica, Mexico and the United States.

Phylogeny of C. lindemuthianum *isolates.* The phylogeny of *C. lindemuthianum* isolates was inferred from the sequence comprising ITS 1 and ITS 2 regions and the 5.8S rRNA gene. The ITS 1 and ITS 2 sequences were aligned to the accessions Z32986 and

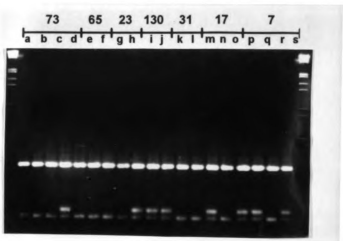

Figure 2.2. PCR amplification products of the ITS1, 5.8S rDNA, and ITS2 region of the rDNA of *C. lindemuthianum* isolates digested with the restriction enzyme *Msp* I, from races 73 (lanes a to d), 65 (lanes e and f), 23 (lanes g and h), 130 (lanes i and j), 31 (lanes k and l), 17 (lanes m to o), 7 (lanes p to s).

Z18975 in the EMBL database (Figure 2.3). The multiple sequence alignment of ITS 1 showed differences among sequences of isolates due to single nucleotide deletions, substitutions or insertions in 25% of the 162 positions. In the ITS 2 region, only single nucleotide insertions were observed in 8% of the 167 positions (Figure 2.3).

The phylogenetic tree derived from the Bootstrap and N-J analyses of the *C. lindemuthianum* isolates comprised a major branch and an outlier race (Figure 2.4). The major branch with a bootstrap value of 88% comprised all races except race 31. Races 23 and 457 formed a cluster with a bootstrap value of 82%, and race 89 with race 2047 formed a cluster supported by a bootstrap value of 75%. The branches combining all other races showed bootstrap values of less than 60%. The phylogenetic tree constructed using the Fitch-Margoliash method is comparable to the tree constructed based on N-J method (Figure 2.5). The outlier race 31 exhibited major differences from the cluster formed by all other isolates.

Parsimony analysis showed similar results to both the N-J and Fitch-Margoliash methods. On the basis of a bootstrap value of 100% all isolates formed a monophyletic group. A subgroup formed by races 457 and 23, also demonstrated by phylogenetic and parsimony analysis was supported by a bootstrap value of 82% and 70%, respectively. Race 31, an outlier in the other phenograms generated by the distance methods, was placed outside the group formed by all other races and was supported by a bootstrap value of 67%.

Table 2.2. Sequence pair distances estimated by the Jukes-Cantor one-parameter method of *C. lindemuthianum* isolates

	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1.000[b]	0.004	0.008	0.015	0.004	0.006	0.008	0.008	0.022	0.002	0.008	0.006	0.011	0.008	0.011
2		1.000	0.013	0.020	0.008	0.002	0.013	0.004	0.026	0.006	0.013	0.011	0.006	0.004	0.006
3			1.000	0.011	0.008	0.015	0.013	0.013	0.022	0.006	0.004	0.006	0.015	0.008	0.015
4				1.000	0.020	0.022	0.020	0.020	0.033	0.017	0.015	0.017	0.022	0.015	0.022
5					1.000	0.011	0.013	0.013	0.022	0.002	0.008	0.002	0.015	0.013	0.015
6						1.000	0.015	0.006	0.029	0.008	0.015	0.013	0.008	0.006	0.008
7							1.000	0.008	0.026	0.011	0.013	0.011	0.011	0.013	0.020
8								1.000	0.026	0.011	0.013	0.011	0.002	0.004	0.011
9									1.000	0.020	0.022	0.020	0.029	0.026	0.029
10										1.000	0.006	0.004	0.013	0.011	0.013
11											1.000	0.006	0.015	0.008	0.020
12												1.000	0.013	0.011	0.017
13													1.000	0.006	0.013
14														1.000	0.011
15															1.000

[a] Races of *C. lindemuthianum*: 1- Arg 81, 2- Bra 89, 3- Bra 31, 4- *Colletotrichum lagenarium* (out group species), 5- Clb 15, 6- CR 2047, 7- DR 38, 8- Mex 457, 9- Mex 73, 10- Per 5, 11- Per 7, 12- Per 8, 13- US 23, 14- US 65, 15- US 73.
Origin of races: Arg (Argentina), Bra (Brazil), Clb (Colombia), CR (Costa Rica), DR (Dominican Republic), Mex (Mexico), Per (Peru), US (United States)
Races identified according to the binary system (Pastor-Corrales, 1991).

[b] Jukes-Cantor one-parameter method was computed by the DNADIST program (PHYLIP) version 3.5c.

→
ITS1

```
ARG81   CTTGTTAAAA TTGGGGGGTT TACGGCAGGA GTGCCCTGCG GGTCCCGGTG CGA
BR89    .......... ........G. .......... .......... .......... ....
BR31    .......... .......... .......... .......... .......... ....
Clag    .....A.... .......... .......... G......... .......... ....
CLB15   .......... .......... .......... .......... .......... ....
CR2047  .......... ........G. .......... .......... .......... ....
DR38    .......... .......... .......... .......... .......... ....
MX457   .......... ........G. .......... .......... .......... ....
MX73    .......... .......... .......... .......... .......... ....
PER5    .......... .......... .......... .......... .......... ....
PER7    .......... .......... .......... .......... .......... ....
PER8    .......... .......... .......... .......... .......... ....
US23    .......... .......... .......... .......... .......... ....
US65    .......... .......... .......... .......... .......... ....
US73    .......... ........G. .......... .......... .......... ....

ARG81   GGTGGTATGT TACTA*CGCA AAGGAGGCTC CGCG*AGGGT CCGCCA*CTG TCTT
BR89    .......... .......... .......... .......... .......... ....
BR31    .......... .......... .......... .......... .......... ....
Clag    .......... .......... .......... ....G..... .......... ....
CLB15   .......... .....A.... .......... .......... .......... ....
CR2047  .......... .......... .......... .......... .......... ....
DR38    .......... .......... .......... .......... .......... ....
MX457   .......... .....A.... .......... .......... .......A... ....
MX73    .......... .......... .......... ....GG..TC .GC.AC.... ....
PER5    .......... .......... .......... .......... .......... ....
PER7    .......... .......... .......... .......... .......... ....
PER8    .......... .......... .......... .......... .......... ....
US23    .......... .......... .......... .......... .......... ....
US65    .......... .......... .......... .......... .......... ....
US73    .......... .......... .......... .....G.... .......... ....

ARG81   T*G*AGGGCC CA*CGTCAGC CGTGG*AAGC CCCAA*CGCC AAGCGG*TGC TTGA
BR89    .......... .......... .......... .......... .......... ....
BR31    .......... .......... .......... .......... .......... ....
Clag    .......... .......... .......... .......... .......... ....
CLB15   .......... .......... .......... .......... .......... ....
CR2047  .......... .......... .......... .......... .......... ....
DR38    .T........ .......... .......... .......... .......... ....
MX457   .T........ ..A....... .......... .....A.... .......... ....
MX73    .......... .......... .......... .....A.... .......... ....
PER5    .......... .......... .......... .......... .......... ....
PER7    .......... .......... .......... .......... .......... ....
PER8    .......... .......... .......... .......... .......... ....
US23    .T.G...... .......... .......... .....A.... ......G... ....
US65    .......... .......... .......... .......... .......... ....
US73    .......... .......... .......... .....A.... .......... ....
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→
ITS3

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ARG81   GGGTTGAAAT GACGCTCGAA CAGGCATGCC CGCCAGAATG CTGGCGGGCG CAAT
BR89    .......... .......... .......... .......... .......... ....
BR31    .......... .......... .......... .......... .......... ....
Clag    .......... .......... .......... .......... .......... ....
CLB15   .......... .......... .......... .......... .......... ....
CR2047  .......... .......... .......... .......... .......... ....
```

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DR38    .......... .......... .......... .......... .......... ....
MX457   .......... .......... .......... .......... .......... ....
MX73    .......... .......... .......... .......... .......... ....
PER5    .......... .......... .......... .......... .......... ....
PER7    .......... .......... .......... .......... .......... ....
PER8    .......... .......... .......... .......... .......... ....
US23    .......... .......... .......... .......... .......... ....
US65    .......... .......... .......... .......... .......... ....
US73    .......... .......... .......... .......... .......... ....

ARG81   GTGCGTTCAA AGATTCGATG ATTCACTGAA TTCTGCAATT CACATTACTT ATCG
BR89    .......... .......... .......... .......... .......... ....
BR31    .......... .......... .......... .......... .......... ....
Clag    .......... .......... .......... .......... .......... ....
CLB15   .......... .......... .......... .......... .......... ....
CR2047  .......... .......... .......... .......... .......... ....
DR38    .......... .......... .......... .......... .......... ....
MX457   .......... .......... .......... .......... .......... ....
MX73    .......... .......... .......... .......... .......... ....
PER5    .......... .......... .......... .......... .......... ....
PER7    .......... .......... .......... .......... .......... ....
PER8    .......... .......... .......... .......... .......... ....
US23    .......... .......... .......... .......... .......... ....
US65    .......... .......... .......... .......... .......... ....
US73    .......... .......... .......... .......... .......... ....

ARG81   CATTTCGCTG CGTTCTTC*A TC*GATGCCA GAACCAAGAG ATCCGTTGTT AAAA
BR89    .......... .......... .......... .......... .......... ....
BR31    .......... .......... .......... .......... .......... ....
Clag    .......... .......... .......... .......... .......... ....
CLB15   .......... .......... .......... .......... .......... ....
CR2047  .......... .......... .......... .......... .......... ....
DR38    .......... .......... .......... .......... .......... ....
MX457   .......... .........C. ..C..... .......... .......... ....
MX73    .......... .......... .......... .......... .......... ....
PER7    .......... .......... .......... .......... .......... ....
PER8    .......... .......... .......... .......... .......... ....
US23    .......... .......... ..C..... .......... .......... ....
US65    .......... .......... .......... .......... .......... ....
US73    .......... .......... .......... .......... .......... ....

            →
          ITS2
ARG81   GTTTTGATTA TTTGCTTGT* GCCACTC*AG AAGAGACGTC GTTAAAATAG AGTT
BR89    .......C.. .......... .......... .......... .......... ....
BR31    .......... .......... .......... .......... .......... ....
Clag    .......... .......... .......... .......... .......... ....
CLB15   .......... .......... .......... .......... .....G.... ....
CR2047  .......C.. .......... .......... ......A... .......... ....
DR38    .......... .......... .......... .......T.. ...*...A.. ....
MX457   .......C.. .........T .......C.. .......... ...T...A.. ....
MX73    .......... .......... .........C. .......... .......... ....
PER5    .......... .......... .......... .......... .......... ....
PER7    .......... .......... .......... .......... .........*. ....
PER8    .......... .......... .......... .......... .......... ....
US23    .......C.. .......... .......C.. .......... ...*...A.. ....
US65    .......C.. .......... .......... .......... .........*. ....
US73    .......C.. .......... .......... .......... .......... ....
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ARG81   GGGTTTTCCT *CCGGCGGGC GCCCC*GCGA GCGGGGCC*G GGGGG*AGG* CGGA
BR89    .......... .......... .......... .......... .......... ....
BR31    .......*.C T......... .....C.... .......A.. .......... ....
Clag    .......T.C T......... T....C.... .........C. .....G...G ....
CLB15   .......... .......... .......... .......A.. .......... ....
CR2047  .......... .......... .......... .......... .......... ....
DR38    .......... .......... .......... A.......A. .......... ....
MX457   .......... .......... .......... .......... .......... ....
MX73    .......... .......... .......... .......A.. .......... ....
PER5    .......... .......... .......... .......A.. .......... ....
PER7    .......... T......... .......... .......A.. .......... ....
PER8    .......... .......... .......... .......A.. .......... ....
US23    .......... T........* .......... .......... ...C...... *...
US65    .......... .......... .....C.... .......... .......... ....
US73    ......C..C .......... .......... .......... .......... ....
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ITS4 ←

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ARG81   CCTCCC*GCC CGCCG*AAGC AACGGTTTGG *TATGTTC*A CAAAGGGTT* ATAGAGG
BR89    .......... .......... .......... .......... .......... .......
BR31    ......C... .........* .......... TA*.....C. .......... .....AC
Clag    .......... .....G.... .......A.. TA......C .........T .....AC
CLB15   .......... .......... .......... .......... .......... .......
CR2047  .......... .......... .......... .......... .......... .......
DR38    .......... .......... .......... T*........ .........T *.....C
MX457   .......... .......... .......... T*........ .......... ......C
MX73    .......... .......... .......... .......... .......... ......C
PER5    .......... .......... .......... .......... .......... .......
PER7    .......... ....*G..*. .......... G......... .......... ...A.AC
PER8    .......... .......... .........G .......... .......... ......C
US23    .......... .......... .......... T*........ .........T *.....C
US65    .......... ....CG..A. .......... .......... .......... .....AC
US73    .......... .......... .......... .......... .......... .......
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Figure 2.3. Sequence alignment (5' - 3' direction) of the rDNA internal transcribed spacer (ITS) sequences and the 5.8S rRNA gene of isolates of *C. lindemuthianum* collected in Arg (Argentina), Bra (Brazil), Clb (Colombia), CR (Costa Rica), DR (Dominican Republic), Mex (Mexico), Per (Peru), and US (United States). The sequence Clag (*Colletotrichum lagenarium*) was used as outgroup in both the phylogenetic and parsimony analysis. (-) indicates identity with Arg81 sequence; (*) indicates an introduced gap. The sequences of the ITS I (positions 1 - 165), the 5.8S rRNA gene (positions 166 - 329), and ITS II (positions 330 - 492) are indicated.

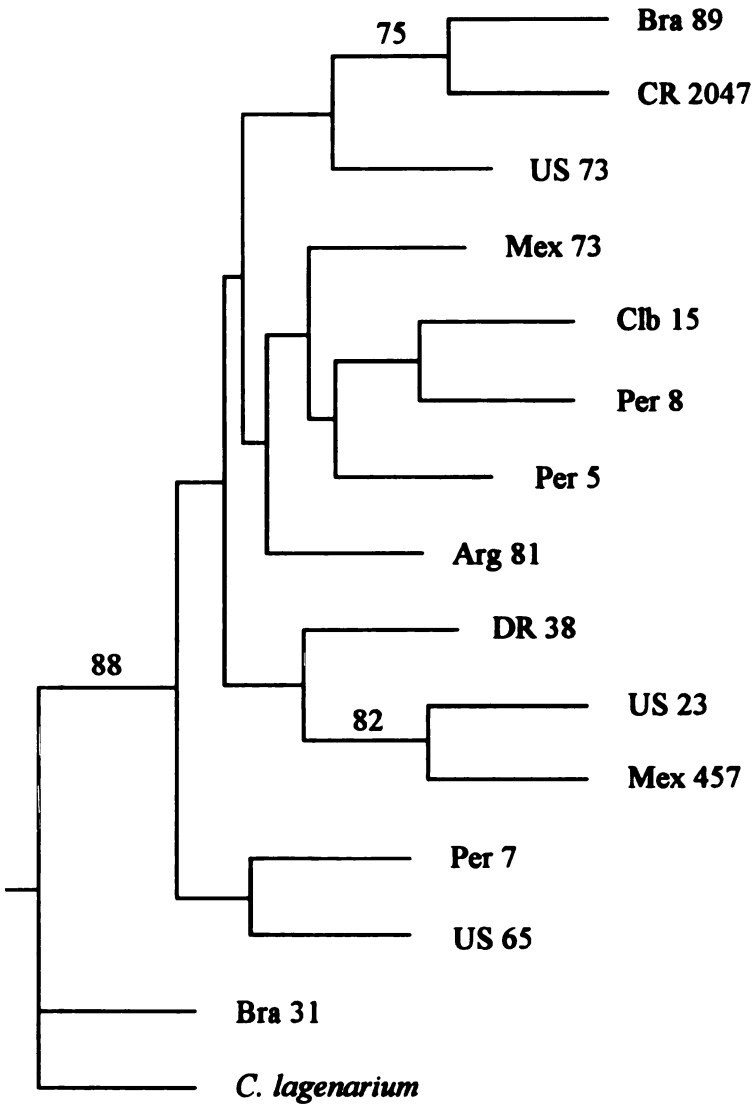

Figure 2.4. Phylogenetic tree indicating the relationships between isolates of *C. lindemuthianum* based on sequences of ITS1, ITS2 and 5.8 rRNA. The tree was created using the neighbor-joining method (NEIGHBOR program in PHYLIP) from distance values estimated by the Jukes-Cantor one-parameter method (DNADIST program in PHYLIP). Confidence limits of the branches, indicated above the line, were created in a bootstrap analysis using 500 replications. Only the bootstrap values above 60% are indicated. *C. lagenarium* was used as outgroup.

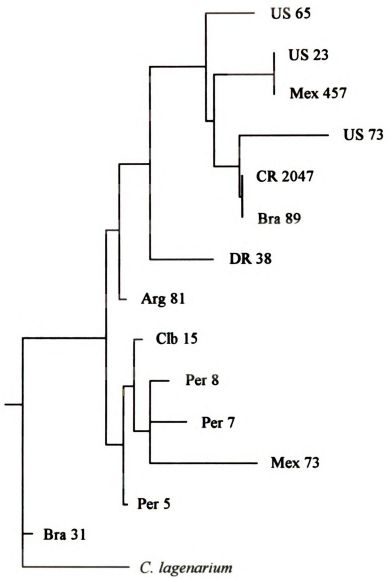

Figure 2.5. Phylogenetic tree indicating the relationships between isolates of *C. lindemuthianum* based on sequences of ITS1, ITS2 and 5.8 rDNA. The tree was created using the Fitch-Margolish method (FITCH program in PHYLIP) from distance values estimated by the Jukes-Cantor one-parameter method (DNADIST program in PHYLIP). *C. lagenarium* was used as outgroup.

Genetic distances among races of *C. lindemuthianum* ranged from 0.2% to 2.9% indicating variable levels of dissimilarity. Sequences did not show pattern parallel to a specific reaction group (Table 2.2). For instance, the Andean race 38 showed homology to the other Andean races ranged from 1.1% to 1.3%, whereas the homology to the Middle American races ranged from 0.8% to 2.6%. The Middle American race 73 from the United States showed homology to the Andean races ranging from 1.3% to 2.0%, whereas the homology to the other Middle American races ranged from 0.6% to 2.9%. Isolates of race 73 showed the greatest genetic distance (2.9%) among races of *C. lindemuthianum*. Likewise, the intra-race dissimilarity was supported by RFLP-ITS (Table 2.1) and phylogenetic analyses (Figure 2.4, 2.5, 2.6). Our data suggest that polymorphism in rDNA is not linked to specific *P. vulgaris* gene pool. In addition, population structure of *C. lindemuthianum* is not structured along with the geographic origin of races.

DISCUSSION

ITS-rDNA has been used to estimate intra- or inter-specific divergence analysis in several fungi species (Nazar et al., 1991; Liu and Sinclair, 1992; Kusaba and Tsuge, 1995; Bunting et al., 1996; Cooke and Duncan, 1997; Fouly et al., 1997; Kropp et al., 1997). The high resolution of sequence analysis of the variable regions within the rDNA has been used to solve taxonomic misclassifications in *Colletotrichum* species (Sherriff et al., 1994; Sreenivasaprasad et al., 1994; Sreenivasaprasad et al., 1996). ITS 1 region within the rDNA region appears to contain most of the divergence among *Colletotrichum*

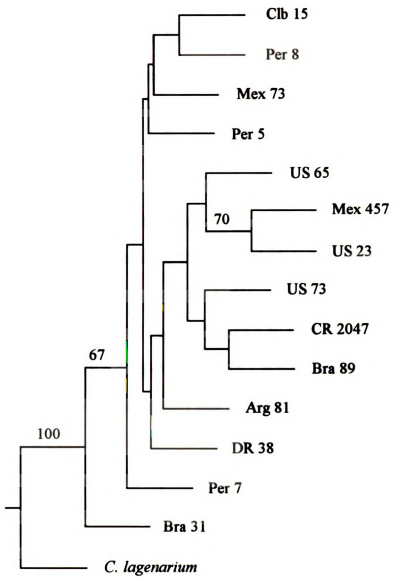

Figure 2.6. Phylogenetic tree indicating the relationships between isolates of *C. lindemuthianum*. The tree was constructed by parsimony analysis of ITS1, ITS2 and 5.8 rDNA sequences. Confidence limits of the branches, indicated above the line, were created in a bootstrap analysis using 500 replications. Only the bootstrap values above 60% are indicated. *C. lagenarium* was used as an outgroup.

species and other fungal species (Sreenivasaprasad et al., 1996; Bunting et al., 1996; Cooke and Duncan, 1997). However, Sherriff et al. (1994) observed closer relationship of *C. lindemuthianum* to *C. malvarum*, *C. orbiculare*, and *C. trifolii* on the basis of the ITS 2 region and the 28S rDNA sequences. The high resolution of sequences within ITS 2 region allowed identification of more than one species among isolates from *C. gloesporioides*. Analysis of both ITS 1 and ITS 2 regions might be more informative if pathogens exhibit high homology within ribosomal genes (Nazar et al., 1991) or in the case of closely related isolates within a species (Faris-Mokaiesh et al., 1996). Most of the variability observed in the sequence analyses of 14 isolates of *C. lindemuthianum* was restricted to the ITS 1 region which exhibited 25% of variability, whereas 8% of variability was present in the ITS 2 region (Figure 2.3). However, the analysis of the region comprising the ITS 1, 5.8S rRNA and ITS 2 in the *C. lindemuthianum* isolates allowed better resolution of some groups than the separate analysis of each ITS region.

Comparison of restriction patterns derived from ITS regions has proved to be a useful molecular approach to classify fungal species (Fouly et al., 1997). RFLP analysis of ITS regions in *Colletotrichum* demonstrated considerable heterogeneity within *C. acutatum* isolated from different hosts (Sreenivasaprasad et al., 1992). Differences in the RFLP-ITS banding patterns of isolates of *C. nympaheae* from Europe compared to isolates infecting the same host in North America supported the naming of *C. nupharicola* as a new species (Johnson et al., 1997).

Some degree of specialization within *C. lindemuthianum* that corresponded to the two host gene pools within *P. vulgaris* (i. e. Middle American and Andean gene pools) was demonstrated by virulence analysis and molecular markers, such as RAPDs and

RFLP-ITS (Fabre et al., 1995; Pastor-Corrales, 1996; Sicard et al., 1997). However, previous work did not show separation of races congruent with specific host gene pool based on virulence and RAPD analyses. Isolates virulent to each gene pool showed high levels of similarity and they were present in all clusters (Balardin et al., 1997). Our data for RFLP-ITS analysis showed no cluster formed exclusively by one reaction group. Andean races clustered predominantly into group I, although the Andean race 23 was observed within the group II (Table 2.1). Middle American races were observed in both groups.

Polymorphism in rDNA-RFLP linked to the geographic origin of isolates has been suggested among *Colletotrichum* spp (Sreenivasaprasad et al., 1992; Johnson et al., 1997). However, sequencing analysis of rDNA of *Puccinia* spp showed no obvious effect of geographic origin on clustering of isolates from Europe and the United States (Kropp, et al., 1997). Our data based on RFLP-ITS and sequencing-ITS did not cluster all isolates from the same country. Bootstrap analysis supported the cluster formed by race 89 from Brazil and race 2047 from Costa Rica. Likewise, the cluster formed by race 23 from the United States and race 457 from Mexico was supported by a bootstrap value of 82% (Figure 2.4). In *C. lindemuthianum*, polymorphisms in rDNA appear not to be linked to the geographic origin of races.

Molecular polymorphism within similar fungal virulence phenotypes has been reported in some fungal species. Kolmer et al. (1995) observed a high degree of molecular polymorphism among isolates that had the same virulence phenotype, in *Puccinia recondita* f.sp. *tritici*. The molecular polymorphism within virulence phenotypes was considered to be a factor that explained the low correlation between

virulence and molecular data. Balardin et al (1997) observed RAPD polymorphism within and among several isolates of ten different races from different countries. Our data based on RFLP-ITS or sequencing-ITS analyses supported these findings. Discrete molecular variations may not be detected by virulence analysis. The distance matrix value, which reflects the divergence in the ITS region, was the highest between isolates of race 73 from Mexico and the United States (2.9%). The intra-race polymorphism observed using either molecular marker suggests a high level of molecular variability within *C. lindemuthianum*. Further analyses might indicate if such intra-race variability is significant for the pathogen population structure.

A monophyletic group of *C. lindemuthianum* races was observed in both the parsimony and phylogenetic analyses. On the basis of bootstrap values, similarity was observed among all races. The only exception was race 31, outlier in the phenograms generated by the distance methods, and placed outside the group formed by all other races. This race showed the lowest genetic divergence from *C. lagenarium* (outgroup species). In addition, high homology was observed between race 31 and the Andean races 5 and 7 from Peru and 15 from Colombia. Interestingly, race 31 was collected from Middle American hosts in Brazil, but showed low homology to the Middle American races 73 from the United States, 89 from Brazil, 457 from Mexico and 2047 from Costa Rica. Race 31 is one of the few races with equivalent high levels of virulence on both Andean and Middle American resistance genes.

Variable level of homology among races and polymorphism in the rDNA region not associated with geographic origin is a clear evidence of the high level of molecular variability within *C. lindemuthianum*. In addition, high molecular homology between

Andean and Middle American races was indicative of no parallel evolution among *C. lindemuthianum* and *P. vulgaris*. These findings disagree with previous work, which showed separation of races of *C. lindemuthianum* congruent to the *P. vulgaris* gene pools (Fabre et al., 1995; Sicard et al., 1997). The intra-race variability emphasized the limitations of virulence analysis. Taking together, our data showed a level of variability within *C. lindemuthianum* higher than that previously characterized by virulence analysis (Balardin et al., 1997). Long-term resistance might be more effective if resistance genes were combined based on their effectiveness against the broader variability of this pathogen defined by both virulence and molecular analyses.

LIST OF REFERENCES

Arora, D. K., Hirsch, P. R., and Kerry, B. R. 1996. PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. Mycol. Res. 100:801-809.

Balardin, R. S. and Kelly, J. D. 1997. Re-characterization of *Colletotrichum lindemuthianum* races. Ann. Rep. Bean Improv. Coop. 40:126-127.

Balardin, R.S., Jarosz, A. M., and Kelly, J.D. 1997. Virulence and molecular diversity in *Colletotrichum lindemuthianum* from South, Central and North America. Phytopathology 87:(in press).

Bunting, T. E., Plumley, K. A., Clarke, B. B., and Hillman, B. I. 1996. Identification of *Magnaporthe poae* by PCR and examination of its relationship to other fungi by analysis of their nuclear rDNA ITS-1 regions. Phytopathology 86:398-404.

Cooke, D. E. L. and Duncan, J. M. 1997. Phylogenetic analysis of *Phytophthora* species baed on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycol. Res. 101:667-677.

Dowling, T. E., Moritz, C., Palmer, J. D., and Rieseberg, L. H. 1996. Nuclei acids III: analysis of fragments and restriction sites. Pages 249-320 in: Molecular Systematics. D.M. Hills, C. Moritz, and B.K. Mable, eds. Sinauer, Sunderland, MA.

Fabre, J. V., Julien, J., Parisot, D., and Dron, M. 1995. Analysis of diverse isolates of *Colletotrichum lindemuthianum* infecting common bean using molecular markers. Mycol. Res. 99:429-435.

Faris-Mokaiesh, S., Boccara, M., Denis, J. B., Derrien, A., and Spire, D. 1996. Differentiation of the "*Ascochyta* complex" fungi of pea by biochemical and molecular markers. Curr. Genet. 29:182-190.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Fouly, H. M., Wilkinson, H. T., and Chen, W. 1997. Restriction analysis of internal transcribed spacers and the small subunit gene of ribosomal DNA among four *Gaeumannomyces* species. Mycologia 89:590-597.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K., and Zimmer, E. A. 1996. Nucleic acids IV: sequencing and cloning. Pages 321-381 in: Molecular Systematics. D.M. Hills, C. Moritz, and B.K. Mable, eds. Sinauer, Sunderland, MA.

Johnson, D. A., Carris, L. M., and Rogers, J. D. 1997. Morphological and molecular characterization of *Colletotrichum nymphaeae* and *C. nupharicola* sp. nov. on water-lilies (*Nymphaea* and *Nuphar*). Mycol. Res. 101:641-649.

Jukes, T. H., and Cantor, C. R. 1969. Evolution of protein molecules. Pages 21-132 In: Mammalian protein metabolism. H. N. Munro, ed. Academic Press, New York.

Kolmer, J. A., Liu, J. Q., and Sies, M. 1995. Virulence and molecular polymorphism in *Puccinia recondita* f.sp. *tritici* in Canada. Phytopathology 85:276-285.

Kropp, B. R., Hansen, D. R., Wolf, P. G., Flint, K. M., and Thomson, S. V. 1997. A study on the phylogeny of the Dyer's woad rust fungus and other species of *Puccinia* from crucifers. Phytopathology 87:565-571.

Kusaba, M., and Tsuge, T. 1995. Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal trascribed spacers of ribosomal DNA. Curr Genet 28:491-498.

Leung, H., Nelson, R. J., and Leach, J. E. 1993. Population structure of plant pathogenic fungi and bacteria. Advances in Plant Pathology 10:157-205.

Liu, Z. L. and Sinclair, J. B. 1992. Genetic diversity of *Rhizoctonia solani* anastomosis group 2. Phytopathology 82:778-787.

McDonald, B. A. 1997. The population genetics of fungi: tools and techniques. Phytopathology 87:448-453.

Milgroom, M. G. 1995. Analysis of population structure in fungal plant pathogens. Pages 213-229 in: Disease analysis through genetics and biotechnology. J. F. Leslie, and R.A. Frederiksen, eds. Iowa State University Press. Ames, IO.

Mills, P. R., Sreenivasaprasad, S., and Brown, A.E. 1992. Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. FEMS Microbiology Letters 98:137-144.

Nazar, R. N., Hu, X., Schmidt, J., Culham, D., and Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. Physiol. and Mol. Plant Pathol. 39:1-11.

Palumbi, S. R. 1996. Nucleic acids II: the polymerase chain reaction. Pages 205-247 in: Molecular Systematics. D. M. Hills, C. Moritz, and B. K. Mable, eds. Sinauer, Sunderland, MA.

Pastor-Corrales, M. A. 1991. Estandarización de variedades diferenciales y de designación de razas de *Colletotrichum lindemuthianum*. Phytopathology 81:694 (abstract).

Pastor-Corrales, M. A. 1996. Traditional and molecular confirmation of the coevolution of beans and pathogens in Latin America. Ann. Rep. Bean Improv. Coop. 39:46-47.

Sicard, D., Michalakis, Y., Dron, M., Neema, C. 1997. Genetic diversity and pathogenic variation of *Colletotrichum lindemuthianum* in the three centers of diversity of its host, *Phaseolus vulgaris*. Phytopathology, 87:807-813.

Sherriff, C., Whelan, M.J., Arnold, G. M., Lafay, J. F., Brygoo, Y., and Bailey, J. A. 1994. Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. Experimental Mycology 18:121-138.

Smith, J. J., Scott-Craig, J. S., Leadbetter, J. R., Bush, G. L., Roberts, D. L., and Fulbright, D. W. 1995. Characterization of random amplified polymorphic DNA (RAPD) products from *Xanthomonas campestris*: implications for the use of RAPD products in phylogenetic analysis. Mol. Phyl. Evol. 3:135-145.

Sreenivasaprasad, S., Brown, A. E., and Mills, P. R. 1992. DNA sequence variation and interrelationships among *Colletotrichum* species causing strawberry anthracnose. Physiol. and Mol. Plant Pathol. 41:265-281.

Sreenivasaprasad , S., Mills, P. R., and Brown, A. E. 1994. Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. acutatum*. Mycol. Res. 98:186-188.

Sreenivasaprasad , S., Mills, P. R., Meehan, B. M., and Brown, A. E. 1996. Phylogeny ans systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. Genome 39:499-512.

Waalwijk, C., de Koning, J. R. A., Baayen, R. P. 1996. Discordant groupings of *Fusarium* spp from sections *Elegans*, *Liseola* and *Dlaminia* based on ribosomal ITS1 and ITS2 sequences. Mycologia 88:361-368.

Chapter 3

INTERACTION AMONG VARIABILITY IN *COLLETOTRICHUM LINDEMUTHIANUM* AND DIVERSITY IN *PHASEOLUS VULGARIS*

ABSTRACT

Thirty-four races of *Colletotrichum lindemuthianum* from Argentina, Brazil, Colombia, Costa Rica, the Dominican Republic, Honduras, Mexico, Peru, and the United States were inoculated on sixty-two cultivars of *Phaseolus vulgaris* from Brazil, the Dominican Republic, Honduras, Mexico, and the United States. Bean genotypes clustered based on the gene pool origin of the resistance genes present, regardless of the actual gene pool of the host genotype. Further sub-groups of cultivars based on overall level of resistance within each gene pool, were observed. Races of *C. lindemuthianum* with Middle American reaction showed broad virulence on germplasm from both gene pools, whereas races with Andean reaction showed high virulence only on Andean germplasm. The reduced virulence of Andean races on Middle American genotypes suggests selection of virulence factors congruent with diversity in *P. vulgaris*. In addition, races of *C. lindemuthianum* were grouped according to specific gene pool (i.e. Middle American and Andean reaction groups) based on principal component analysis. However, the overlapping of specific races with races from different reaction groups might indicate that this group of isolates possesses factors of virulence to both host gene pools. Similar results from phenetic analyses showed races grouped according to specific gene pool.

Most races with Andean reaction were observed in our cluster B, except races 15 and 23, which clustered with Middle American races. Only races 38, 39, and 47 from the Dominican Republic showed high similarity in both multivariate analyses, and clustered based on geographic effect. Data based on virulence supports variability in *C. lindemuthianum* structured with diversity in *Phaseolus*.

INTRODUCTION

Anthracnose, caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams. –Scrib. is an endemic and more severe disease in Central America than in Andean South America or in the temperate regions of North America and Europe (Pastor-Corrales et al., 1994). Yield losses from anthracnose can be as high as 95% (Guzman et al., 1979). Control of this disease is difficult due to the efficient seed transmission of the pathogen (Tu, 1992), lack of cost-effective chemical controls (Pastor-Corrales and Tu, 1989), ability of *C. lindemuthianum* to survive in plant debris up to 22 months (Dillard and Cobb, 1993), and eventual development of sclerotia (Sutton, 1992). Although the best approach to controlling this disease is through an integrated pest management regime (IPM), host resistance seems to be more appropriate in countries where implementation of IPM measures is not feasible. However, the magnitude of variability in the *C. lindemuthianum* reported worldwide (Andrus and Wade, 1942; Barrus, 1918; Blondet, 1963; Burkholder, 1923; Fouilloux, 1979; Menezes and Dianese, 1988; Garrido, 1986; Balardin et al., 1990; Waterhouse, 1975), has been the major limitation for developing durable resistance in commercial germplasm.

The gene-for-gene system regulates the interaction among *P. vulgaris* and *C. lindemuthianum*. Evolving races of *C. lindemuthianum* continue to overcome specific resistance sources. Prior to the appearance of delta race of *C. lindemuthianum*, the *Co-1* gene was the only resistance source present in navy beans in North America (Tu, 1988). The *Co-2* gene, which has conferred resistance to races 17, 130, 102, 23, 65 and 55, was the predominant resistance source used in Europe and North America (Mastenbroek,

1960; Kruger et al., 1977; Fouilloux, 1979). However, identification of races 31, 63, and 89 from isolates collected in Brazil and Europe (Kruger et al., 1977; Fouilloux, 1979), necessitated the identification and characterization of new resistance sources. Three resistance genes, *Co-4*, *Co-5*, and *Co-3*, from a Mexican germplasm collection were characterized. The *Co-4* and *Co-5* genes conferred resistance to the kappa, iota and alpha-Brazil races, whereas the *Co-3* gene was susceptible to the race alpha-Brazil (Fouilloux, 1979).

Long term resistance based on major genes has been ineffective when resistance genes are deployed one at a time (Duvick, 1996). For instance, the bean genotypes TO (*Co-4*), PI 207262, and Mexico 222 (*Co-3*), reported to be resistant to European and North American races (Kruger et al., 1977; Fouilloux, 1979; Schwartz et al., 1982), were susceptible to Latin American races (Menezes and Dianese, 1988; Rava et al., 1993; Rodriguez, 1991; Restrepo, 1994; Pastor-Corrales et al., 1995). Isolates from Honduras overcame resistance in the cultivars TU (*Co-5*) and AB 136 (*Co-6*) (Balardin et al., 1997), which have been used as parents in various breeding programs in Latin America. The development of complementary resistance genes to counter most pathogen variability (Duvick, 1996; Casela et al., 1996; Levy et al., 1993), and pyramiding resistance genes from gene pools other than from which the breeding germplasm has been selected was suggested as an efficient approach to developing durable anthracnose resistance (Young and Kelly, 1997).

Variability in *Uromyces phaseoli* (Stavely, 1982; Stavely, 1984; Maclean et al., 1995), *Phaeoisariopsis griseola* (Guzman et al., 1995), and *C. lindemuthianum* (Fabre et al., 1995; Pastor-Corrales, 1996; Sicard et al., 1997) congruent with *P. vulgaris*

germplasm diversity in the Middle American and South American Andean gene pools was suggested as an evolutionary event occurring among host and pathogen populations. As a result, reciprocal selection of resistance genes in *P. vulgaris* and virulence genes in *C. lindemuthianum* might have occurred (Gepts, 1988). In this study our objective was to determine the reaction of germplasm from Brazil, Honduras, Mexico and the United States to a representative group of races of *C. lindemuthianum* from South, Central and North America. Virulence tests and multivariate analyses were used to demonstrate the effect of *P. vulgaris* gene pool on the population structure of the anthracnose pathogen and the reciprocal effect of the pathogen on the same host cultivars. Breeding strategies to improve durable resistance to *C. lindemuthianum* based on these findings is discussed.

MATERIALS AND METHODS

Phaseolus vulgaris *germplasm and* Colletotrichum lindemuthianum *races.* Sixty-two genotypes of *P. vulgaris* were inoculated with thirty-four races of *C. lindemuthianum.* Genotypes were divided into two groups: a) 50 cultivars from Brazil, the Dominican Republic, Honduras, Mexico, and the United States, and b) the 12 anthracnose-differential cultivars (Pastor-Corrales, 1991) which were used as control and inoculated to verify the identity of a particular race. The 34 races of *C. lindemuthianum* were identified from isolates collected in Argentina, Brazil, Colombia, Costa Rica, the Dominican Republic Honduras, Mexico, Peru, and the United States. Identification, origin and characteristics of the genotypes is shown in Table 3.1. The gene pool of

Table 3.1. Identification, origin, gene pool, race, seed weight, and resistance index of common bean genotypes (*P. vulgaris* L.). Genotypes were from Brazil, Colombia, the Dominican Republic, the Netherlands, Honduras, Mexico and the United States and were inoculated with 34 races of *C. lindemuthianum* from Argentina, Brazil, Colombia, Costa Rica, the Dominican Republic, Honduras, Mexico, Peru, and United States.

Genotypes[a]	Origin[b]	GP[c]	Race[d]	SW[e]	RI[f]
1 - MDRK	US	A	Nueva Granada	34.9	44
2 - Perry Marrow	US	A	Nueva Granada	44.2	59
3 - Charlevoix	US	A	Nueva Granada	53.0	59
4 - Seafarer	US	M	Meso America	17.2	47
5 - Montcalm	US	A	Nueva Granada	54.0	67
6 - Red Hawk	US	A	Nueva Granada	56.0	56
7 - Widusa	Nth	M	Meso America	15.6	70
8 - Pinto Villa	Mex	M	Durango	37.2	67
9 - Bayo Victoria	Mex	M	Durango	44.0	67
10 - Isles	US	A	Nueva Granada	62.0	76
11 - Kaboon	Nth	A	Nueva Granada	36.1	79
12 - Pompadour Checa 50	DR	A	Nueva Granada	44.7	67
13 - Ruddy	US	A	Nueva Granada	52.0	56
14 - Cacahuate 72	Mex	A	Nueva Granada	43.5	82
15 - Chihuahua 21	Mex	A	Nueva Granada	35.5	6
16 - Bayomex	Mex	A	Nueva Granada	22.5	6
17 - Isabella	US	A	Nueva Granada	51.0	0
18 - Cardinal	US	A	Nueva Granada	62.0	3
19 - Taylor Horticultural	US	A	Nueva Granada	54.0	3
20 - G 2333	Mex	M	Meso America	18.8	100
21 - SEL 1308	US	M	Meso America	24.0	97
22 - Catrachita	Hon	M	Meso America	31.2	94
23 - BAT 93	US	M	Meso America	17.0	85
24 - SEL 1360	US	M	Meso America	24.0	97
25 - TO	Mex	M	Meso America	28.4	73
26 - AB 136	Mex	M	Meso America	23.8	91

Table 3.1 (cont'd)

27 - PI 207262	Mex	M	Meso America	20.2	79
28 - FM M38	Mex	M	Jalisco	28.2	82
29 - Yeguare	Hon	M	Meso America	19.0	79
30 - Macanudo	Bra	M	Meso America	23.3	65
31 - Negro 150	Mex	M	Jalisco	29.0	62
32 - Zacatecas 15	Mex	M	Durango	14.5	67
33 - TU	Mex	M	Meso America	21.2	88
34 - Blackhawk	US	M	Meso America	19.0	67
35 - Newport	US	M	Meso America	25.0	59
36 - Cornell 49242	US	M	Meso America	17.7	65
37 - Mexico 222	Mex	M	Meso America	25.1	59
38 - Amarillo de Calpan	Mex	M	Jalisco	27.5	47
39 - Rio Tibagi	Bra	M	Meso America	21.0	51
40 - Amarillo 169	Mex	M	Jalisco	21.7	47
41 - Flor de Mayo	Mex	M	Jalisco	22.8	23
42 - Puebla 36	Mex	M	Jalisco	24.0	35
43 - C-20	US	M	Meso America	18.4	41
44 - Michoacan 8-A	Mex	M	Jalisco	27.5	65
45 - Durango 32	Mex	M	Durango	20.0	42
46 - Gilguerillo	Mex	M	Jalisco	24.5	38
47 - Carioca	Bra	M	Meso America	18.5	38
48 - Criollo Negro	Mex	M	Meso America	26.8	35
49 - MD 2324	Hon	M	Meso America	20.5	38
50 - Tio Canela 75	Hon	M	Meso America	17.5	15
51 - Schooner	US	M	Meso America	19.0	23
52 - Bayo Berrendo	Mex	M	Meso America	17.5	17
53 - MD 3037	Hon	M	Meso America	22.8	20
54 - Azufrado	Mex	M	Durango	28.0	41
55 - T-39	US	M	Meso America	20.0	23
56 - Negro Durango	Mex	M	Durango	32.5	17
57 - Dorado	Hon	M	Meso America	23.0	26

58 - Desarrural IR	Hon	M	Meso America	17.0	3
59 - Danli 46	Hon	M	Meso America	22.8	17
60 - Zamorano	Hon	M	Meso America	20.0	6
61 - FT 83-120	Bra	M	Meso America	22.2	26
62 - Michelite	US	M	Meso America	16.4	15

[a] Seed provided by: Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), Brazil/RS; Escuela Agricola Panamericana Zamorano, Honduras; National Research Institute for Forestry and Agriculture (INIFAP), Mexico; Centro Internacional de Agricultura Tropical (CIAT), Colombia; Bean Breeding and Genetics Program (Michigan State University), United States.

[b] Origin of cultivars: Bra (Brazil), Clb (Colombia), DR (Dominican Republic), Hon (Honduras), Mex (Mexico), Nth (Netherlands), US (United States).

[c] Andean (A) and Middle American (M) gene pools of *P. vulgaris* (Singh et al., 1991).

[d] Races of *P. vulgaris* (Singh et al., 1991).

[e] Seed weight (g/100 seeds).

[f] Resistance Index: (total no. of resistance reactions / 34), 34 being the total number of races used as inoculum in this trial.

genotypes was inferred based on previous knowledge of host cultivars. Identification, reaction group and origin of all races is shown in Table 3.2.

Determination of a genotype reaction. Inoculum of *C. lindemuthianum* was increased in modified Mathur's medium. The culture medium was prepared with dextrose (8 $g.l^{-1}$), $MgSO_4.7H_2O$ (2.5 $g.l^{-1}$), KH_2PO_4 (2.7 $g.l^{-1}$), neopeptone (2.4 $g.l^{-1}$), yeast extract (2.0 $g.l^{-1}$), and agar (16 $g.l^{-1}$). Spore suspensions for seedling inoculation were prepared from purified single-conidial isolates by flooding plates with 5 ml of 0.01% Tween 80 in distilled water. After scraping the culture surface with a spatula, the dislodged spores were filtered through cheesecloth. The spore concentration was adjusted to 1.0×10^6 $spores.ml^{-1}$ with a hemocytometer.

Seeds for each of the sixty-two *P vulgaris* genotypes were planted in flats containing Baccto planting mix (Michigan Peat Co., Houston TX), and grown under greenhouse conditions (16-h day length at 25°C), for 7 to 10 days until seedlings had reached the full expanded primary leaf stage. Six seedlings were spray-inoculated with the standardized spore suspension. The suspension of inoculum was applied until runoff on the stem and to both surfaces of the unifoliolate leaves. After inoculation, plants were maintained in high humidity (>95%) for 48 h at 22 to 25°C. Plants were allowed to dry and were then transferred to greenhouse benches for 5 days. Disease reaction was rated seven days after inoculation based on a 1 to 9 severity scale (Balardin et al., 1990). Plants with no visible disease symptoms or only a few, very small lesions mostly on primary leaf veins were recorded as resistant (scale 1 to 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 (scale 3.1 to 9). Intermediate reactions (3.1-6.9) were considered as susceptible for the purpose of this work.

Data analysis. Virulence index of each race of *C. lindemuthianum* was computed using the number of cultivars with susceptible reaction over the 62 inoculated genotypes (15 from the Andean gene pool, 47 from the Middle American gene pool). The resistance index of each genotype was computed using the number of cultivars, which exhibited resistance reaction divided by 34. The total number of inoculated races was 34 (Table 3.1).

Principal component analysis was used to derive the variance from severity data associated with the first three principal components. A correlation matrix of severity data obtained from inoculation of 34 races of *C. lindemuthianum* on 62 genotypes was computed by the INTERVAL program in the Numerical Taxonomy and Multivariate Analysis System for personal computer (NTSYS-pc) version 1.70 (Exeter Software, Setauket NY). The eigenvalue and eigenvector matrices were derived from the correlation matrix by the EIGENVECTOR program (NTSYS-pc). The PROJECTION program (NTSYS-pc) projected t severity data from the severity data matrix onto k=3 axes to express the coordinate of each severity datum relative to the axes. The MXPLOT and MOD-3D programs (NTSYS-pc) were used to generate the three-dimensional graphics.

The Phenetic analyses of virulence data was based on a data matrix generated by scoring resistant reaction as 0, and both intermediate and susceptible reactions as 1. Similarity matrices for virulence data were derived with the similarity for genetic data in

the SIMGEN program (NTSYS-pc). Genetic differences between individuals within both *P. vulgaris* and *C. lindemuthianum* populations was calculated using the NEI72 coefficient. Polymorphisms were related to both the accumulated number of gene differences per locus and the proportion of common genes among individuals within populations (Nei, 1972). Cluster analysis was performed using the unweighted pair-group method (UPGMA) in the SAHN program (NTSYS-pc). Phenograms for virulence data were produced using the TREE DISPLAY program (NTSYS-pc).

RESULTS

Andean races of *C. lindemuthianum* exhibited more than 70% virulence on Andean *P vulgaris* germplasm and less than 30% on Middle American germplasm. In contrast, Middle American races of the pathogen had similar virulence indexes on both Andean (55%) and Middle American (60%) germplasm (Table 3.2). Middle American races 31, 87, 357 and 2047 showed a virulence index above 80% on Andean germplasm, whereas only races 1993 and 2047 exhibited a similar virulence index on Middle American germplasm.

The principal component analysis of 62 genotypes of *P. vulgaris* inoculated with 34 races of *C. lindemuthianum* grouped the germplasm on the basis of gene pool and each gene pool was subgrouped according to its overall resistance levels to *C. lindemuthinaum* (Figure 3.1). Four distinct clusters were observed. Cluster A was the most diffuse and included genotypes from both gene pools, although the majority of genotypes were from the Andean gene pool. Cluster B consisted of genotypes exclusively from Andean origin, whereas clusters C and D were comprised of genotypes from the Middle American gene

Table 3.2. Identification, origin, reaction group, and virulence index of 34 races of *C. lindemuthianum*

Race			Virulence Index								
			Germplasm[c]				Differentials[f]				Total[i]
Identification[a]	Origin	RG[b]	A[d]		MA[e]		A[g]		MA[h]		
			No.	%	No.	%	No.	%	No.	%	%
2	Mexico	A	9	60	8	17	1	33	0	0	27
3	Peru	A	11	73	12	26	1	33	1	11	37
5	Peru	A	8	53	10	21	1	33	1	11	29
7	United States	A	12	80	16	34	2	67	1	11	45
15	Colombia	A	13	87	29	62	2	67	2	22	68
19	Dominican Republic	A	9	60	13	28	1	100	2	22	35
23	United States	A	12	80	28	60	2	67	2	22	65
38	Dominican Republic	A	14	93	13	28	3	100	0	0	44
39	Dominican Republic	A	15	100	20	43	3	100	1	11	56
47	Dominican Republic	A	15	100	17	36	3	100	2	22	52
55	Dominican Republic	A	15	100	10	21	3	100	2	22	40
102	United States	A	11	73	8	17	3	100	1	11	31
130	United States	A	10	67	9	19	1	33	1	11	31
1	Brazil	M	4	27	19	40	0	0	1	11	37
8	Peru	M	8	53	18	38	0	0	1	11	42

Table 3.2 (cont'd)

9	**Honduras**	**M**	6	40	25	53	0	0	2	22	50
17	**Brazil**	**M**	8	53	28	60	0	0	2	22	58
31	**Brazil**	**M**	13	87	27	57	2	67	3	33	65
65	**United States**	**M**	8	53	31	66	0	0	2	22	63
73	**United States**	**M**	7	47	25	53	0	0	3	33	52
81	**Argentina**	**M**	2	13	17	36	0	0	3	33	31
87	**Brazil**	**M**	14	93	33	70	2	67	3	33	76
89	**Brazil**	**M**	7	47	27	57	0	0	3	33	55
257	**Mexico**	**M**	8	53	27	57	0	0	2	22	56
321	**Mexico**	**M**	7	47	27	57	0	0	3	33	55
337	**Brazil**	**M**	10	67	28	60	0	0	4	44	61
357	**Mexico**	**M**	12	80	32	67	1	33	4	44	71
453	**Brazil**	**M**	10	67	34	72	1	33	4	44	71
449	**Mexico**	**M**	8	51	35	74	0	0	4	44	68
457	**Mexico**	**M**	8	51	31	67	0	0	5	56	63
521	**Honduras**	**M**	6	40	19	40	0	0	3	34	40
1673	**Honduras**	**M**	6	40	28	59	0	0	5	56	54
1993	**Honduras**	**M**	9	60	44	94	0	0	7	78	85
2047	**Costa Rica**	**M**	15	100	45	96	3	100	8	89	97

[a]Designation of races based on the binary nomenclature system (Pastor-Corrales, 1991), races previously designated by Greek letters: 17 - alpha (α), 19 – epsilon Kenya (εK), 23 – delta (δ), 32 – kappa (κ), 55 - lambda (λ), 65 - epsilon (ε), 81 – eta (ξ), 87 - mu (μ), 89 - alpha-Brazil (αB), 102 – gamma (γ), 130 – beta (β), 453 - zeta (ζ) (Balardin and Kelly, 1997).
[b] Reaction group: Andean and Middle American isolates classified according to the origin and host.
[c] Germplasm Virulence Index (GVI)
[d] GVI of Andean (A) germplasm = total no. of A susceptible genotypes / 15 A genotypes (Table 3.1)
[e] GVI of Middle American (M) germplasm = total no. of M susceptible genotypes / 47 M genotypes (Table 3.1)
[f] Differential cultivars Virulence Index (DVI)
[g] DVI of Andean (A) differential cultivars = total no. of A susceptible differential cultivars / 15 A differential cultivars (Table 3.1)
[h] DVI of Middle American (M) differential cultivars = total no. of M susceptible differential cultivars / 47 M differential cultivars (Table 3.1)
[i] Total Virulence Index: total no. of susceptible genotypes / 62 genotypes.

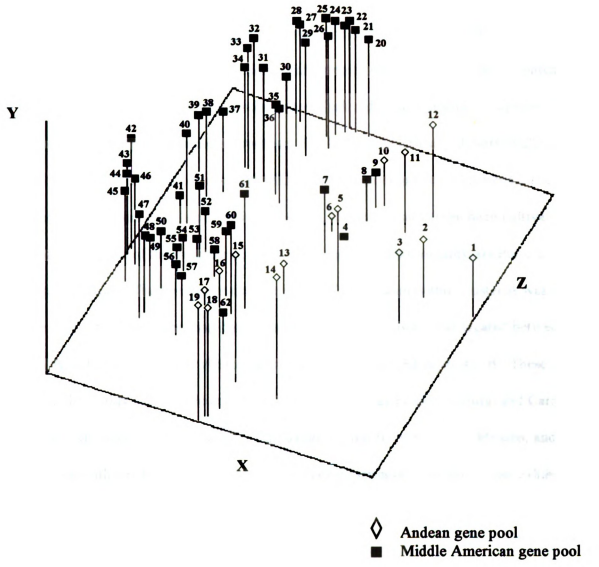

Figure 3.1. Three-dimensional representation of the variance of severity data from inoculation of 34 races of *C. lindemuthianum* on 62 germplasm of *P. vulgaris* associated with the first three principal components. The X-, Y- and Z-axis are the first, second and third principal components, respectively. Germplasm was grouped within Cluster A: 1 to 13; B: 14 to 19; C: 20 to 36; and D: 37 to 62. Identification of cultivars is according to Table 3.1.

pool. The first three principal component axes accounted for approximately 30% of the total variation within the 34 x 34 data matrix.

In cluster A, Andean genotypes included three members of the differential series, MDRK, Perry Marrow and Kaboon, along with three contemporary cultivars, Montcalm, Pompadour Checa and Charlevoix, and two new dark red kidney cultivars, Isles and Redhawk, bred for resistance to anthracnose. The Middle American cultivars in cluster A were located close to the more resistant Middle American members of cluster C. These include the Michigan navy bean cultivar Seafarer, the European garden bean cultivar Widusa (a member of the differential series), and the two Mexican cultivars Pinto Villa and Bayo Victoria. The average resistance index of germplasm within cluster A was 55% (Table 3.1). The Andean cultivar Ruddy, an outlier in this cluster, was located between cluster A and cluster D. Only Andean genotypes were observed in cluster B. These included the highly susceptible cranberry bean cultivar, Taylor Horticultural and Cardinal from the United States, Cacahuate 72, Chihuahua 21 and Bayomex from Mexico, and the kidney bean cultivar Isabella from the United States. Germplasm in this cluster exhibited a 5.5% level of resistance (Table 3.1). Cluster C was formed exclusively by Mesoamerican genotypes that were either members of the differential series or contemporary cultivars bred for resistance to anthracnose. Germplasm within cluster C exhibited 78% level of resistance (Table 3.1). Some genotypes showed resistance levels over 80%, such as G 2333 (100%), breeding lines SEL 1308 and SEL 1360 (97%), Catrachita (94%) and AB 136 (91%). Cultivars G 2333, TO, TU, PI 207262, and Cornell 49242, members of the differential series, fell within this cluster. Cluster D was formed by Mesoamerican genotypes, which were either the most susceptible member of the

differential series, older landraces, traditional or modern cultivars bred for characteristics other than anthracnose. In cluster D, genotypes showed a mean level of resistance of 30% (Table 3.1). Within this group, some traditional cultivars such as Danli 46, Desarrural IR, Dorado, and Zamorano from Honduras, Carioca and Rio Tibagi from Brazil, and the cultivars Schooner, T-39 and C-20 from the United States, were present. The most susceptible differential cultivars Michelite and Mexico 222, were also observed within cluster D.

The principal component analysis of the 34 races of *C. lindemuthianum* is shown in Figure 3.2. The first three principal component axes accounted for approximately 20% of the total variation within the 62 x 62 data matrix. Four distinct clusters are observed. Clusters A and D were comprised of races exhibiting the Middle American and Andean reaction, respectively. Cluster B showed a predominance of races with Middle American reaction along with the Andean races 23 and 15. In contrast, cluster C showed a predominance of races with Andean reaction along with the Middle American race 8. Races within the clusters A, B, and C came from Argentina, Brazil, Honduras, Mexico, Peru, and the United States (Table 3.2), whereas races in cluster D came exclusively from the Dominican Republic. The average virulence indexes of races within clusters A, B, C, and D was 55%, 70%, 35%, and 51%, respectively (Table 3.2).

In cluster A, no race was virulent to any Andean resistance source present in the differential series. However, these races showed 41% of virulence on Andean germplasm from the Dominican Republic, Mexico, and the United States. Races within clusters B and C were virulent to resistance sources from both gene pools. Races from these groups showed a wide range in virulence (53% to 100%) on all genotypes. In cluster D, races 38,

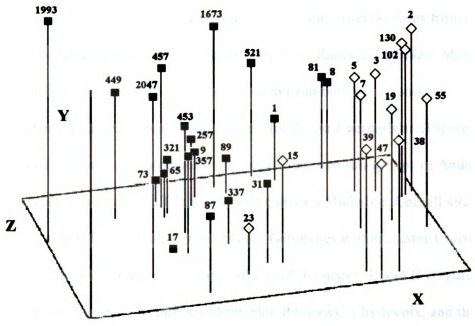

Figure 3.2 Three-dimensional representation of the variance of severity data from inoculation of 34 races of *C. lindemuthianum* on 62 genotypes of *P. vulgaris* associated with the first three principal components. The X-, Y- and Z-axis are the first, second and third principal components, respectively. Origin of races: Bra (Brazil), Clb (Colombia), CR (Costa Rica), DR (Dominican Republic), Hon (Honduras), Mex (Mexico), Per (Peru), US (United States). Races were grouped within clusters A: 1673Hon, 521Hon, 1Bra, 81Bra, 9Hon, 89Bra, 73US, 457Mex, 257Mex, 449Mex, 321Mex, 65US, 1993Hon; B: 453Bra, 17Bra, 337Bra, 357Mex, 2047CR, 31Bra, 15Clb, 23US, 87Bra; C: 8Per, 5Per, 3Per, 2Mex, 130US, 102US, 7US, 55DR, 19DR; and D: 39DR, 38DR, 47DR.

39, and 47 were virulent to all Andean sources present in the differential series. Races from Honduras showed only a Middle American reaction, whereas races from the Dominican Republic showed only an Andean reaction. Races from Brazil, Mexico, Peru, and the United States were virulent on germplasm from both gene pools.

Phenetic analyses showed three clusters for the host germplasm (Figure 3.3). In cluster I Middle American genotypes predominate, but two sub-groups of Andean genotypes were also observed. The differential cultivars Michelite, Cornell 49242, Mexico 222, and TO were placed in this group. Genotypes within cluster I corresponded to those with the lowest level of resistance observed in clusters B and D (Figure 3.1). Exceptions were the Andean cultivars Montcalm, Redhawk, Charlevoix, and the Middle American cultivar Seafarer (Figure 3.1), which corresponded to the highest resistance level within cluster A. Likewise, the cultivars Cornell 49242, Blackhawk, Zacatecas, Negro 150 and Flor de Mayo M38 which showed high levels of resistance grouped in the same region of cluster 1 (Table 3.1). Andean germplasm predominated in cluster II. Interestingly, the highly resistant Middle American breeding lines SEL 1308 and SEL 1360 showed similarity to MDRK. In addition cultivars Bayo Victoria, Pinto Villa and Widusa were placed in cluster II. Although, these cultivars exhibit characteristics of Middle American germplasm, the resistance they possess is derived from Andean sources.

Two major clusters of races corresponding to the Andean and Middle American reaction groups were observed in Figure 3.4. Clusters I and II were 68% dissimilar. In cluster I, Middle American races predominated along with the Andean races 15 and 23. Races with Andean reaction, exclusively formed cluster II. Phenetic analyses showed

Figure 3.3 Phenogram of 62 genotypes of *P. vulgaris* based on virulence data obtained from the inoculation of 34 races of *C. lindemuthianum*. The NEI72 coefficient (SIMGEN - NTSYS-pc) generated a genetic similarity matrix of virulence data. SAHN program (NTSYS-pc) estimated the genetic distances using UPGMA.

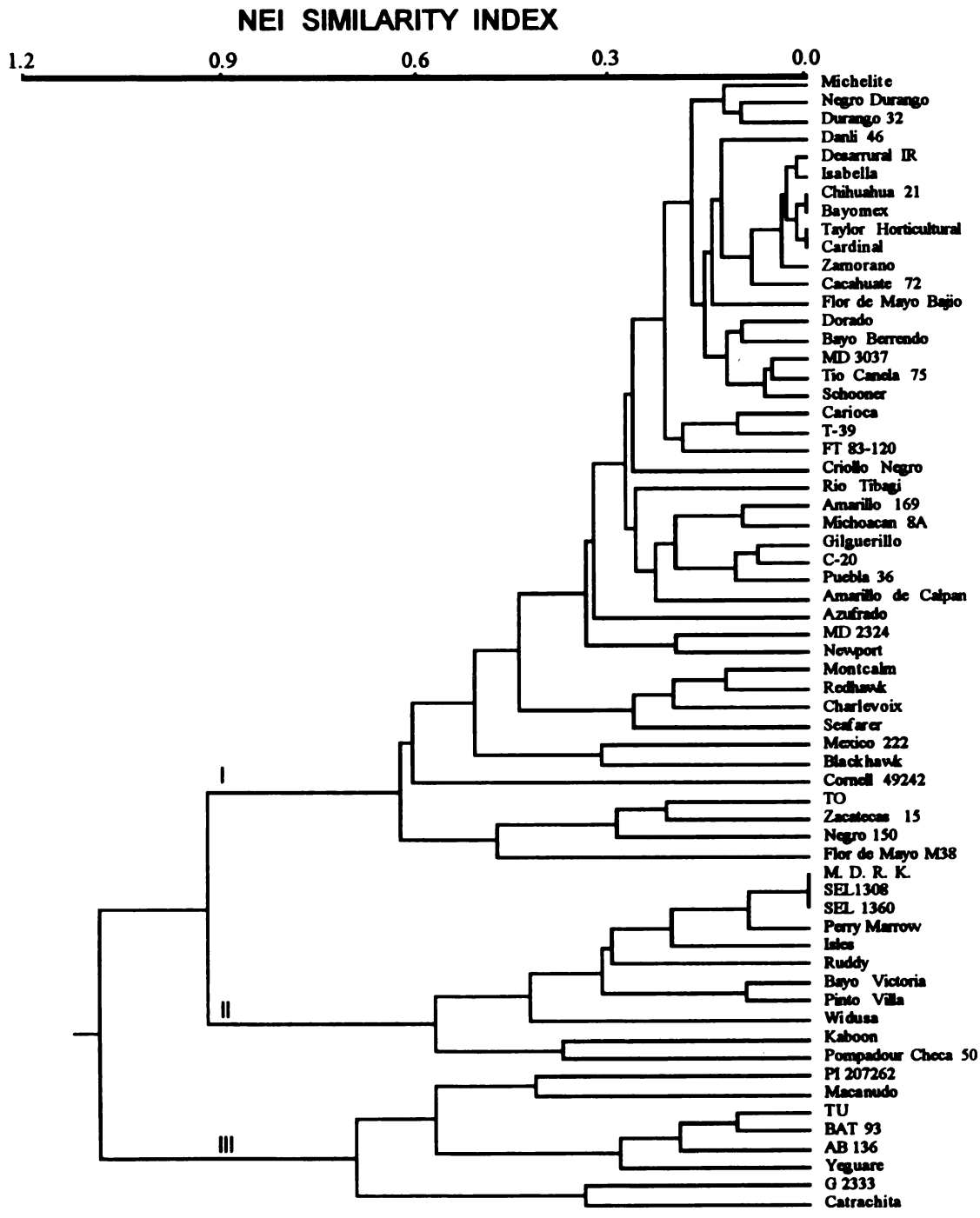
NEI SIMILARITY INDEX
1.2
0.9
0.6
0.3
0.0
Michelite
Negro Durango
Durango 32
Danli 46
Desarrural IR
Isabella
Chihuahua 21
Bayomex
Taylor Horticultural
Cardinal
Zamorano
Cacahuate 72
Flor de Mayo Bajio
Dorado
Bayo Berrendo
MD 3037
Tio Canela 75
Schooner
Carioca
T-39
FT 83-120
Criollo Negro
Rio Tibagi
Amarillo 169
Michoacan 8A
Gilguerillo
C-20
Puebla 36
Amarillo de Calpan
Azufrado
MD 2324
Newport
Montcalm
Redhawk
Charlevoix
Seafarer
Mexico 222
Blackhawk
Cornell 49242
TO
Zacatecas 15
Negro 150
Flor de Mayo M38
M. D. R. K.
SEL1308
SEL 1360
Perry Marrow
Isles
Ruddy
Bayo Victoria
Pinto Villa
Widusa
Kaboon
Pompadour Checa 50
PI 207262
Macanudo
TU
BAT 93
AB 136
Yeguare
G 2333
Catrachita
I
II
III

Figure 3.3

Figure 3.4. Phenogram of 34 *C. lindemuthianum* races based on virulence data obtained from the inoculation on 62 genotypes of *P. vulgaris*. The NEI72 coefficient in the (SIMGEN - NTSYS-pc) generated a genetic similarity matrix of virulence data. SAHN program (NTSYS-pc) estimated the genetic distances using UPGMA. The binary identification of *C. lindemuthianum* races (Pastor-Corrales, 1991) was followed by the origin of isolates: Bra (Brazil), Clb (Colombia), CR (Costa Rica), DR (Dominican Republic), Hon (Honduras), Per (Peru), Mex (Mexico), and US (United States).

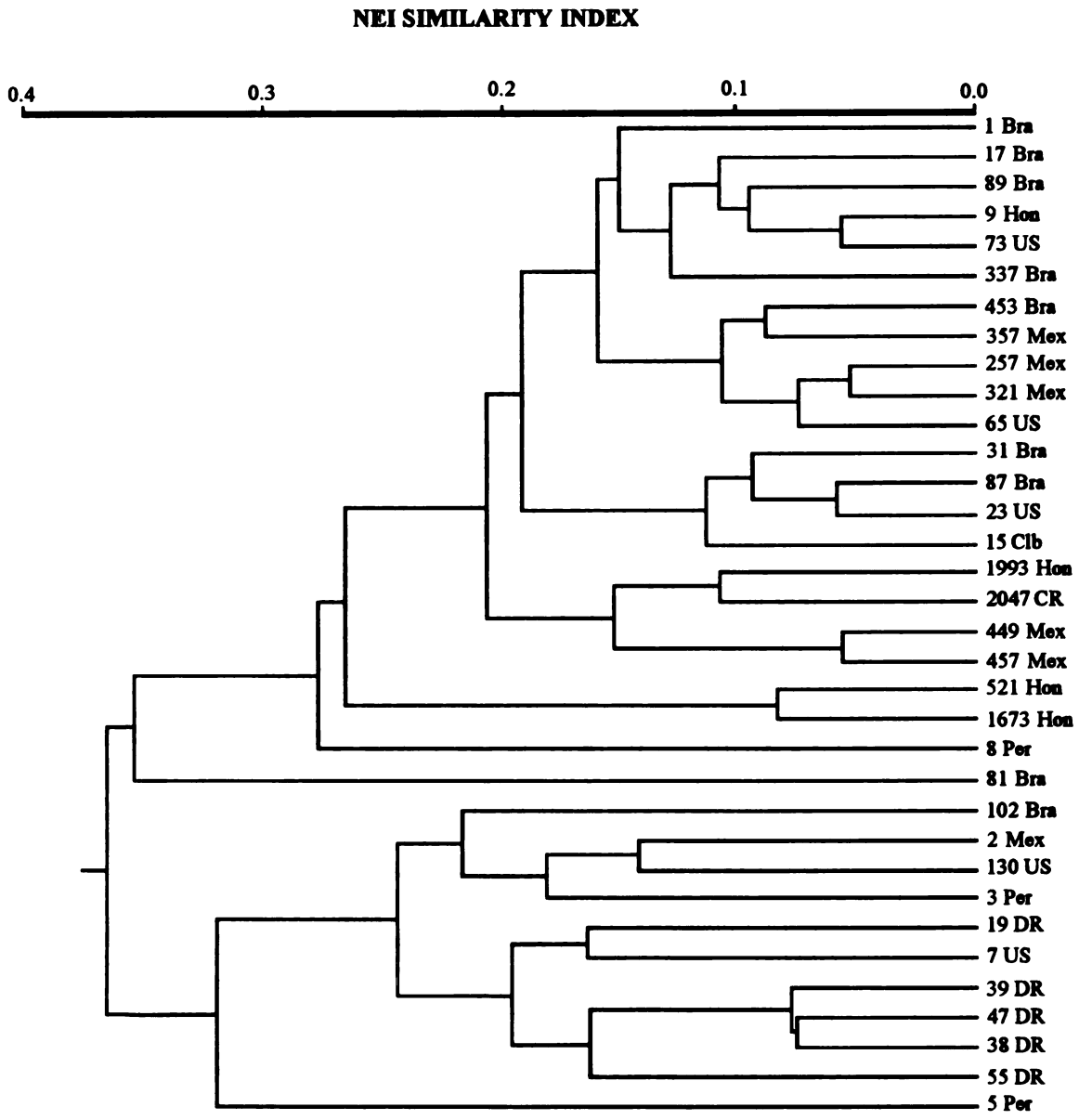
NEI SIMILARITY INDEX
0.4
0.3
0.2
0.1
0.0
1 Bra
17 Bra
89 Bra
9 Hon
73 US
337 Bra
453 Bra
357 Mex
257 Mex
321 Mex
65 US
31 Bra
87 Bra
23 US
15 Clb
1993 Hon
2047 CR
449 Mex
457 Mex
521 Hon
1673 Hon
8 Per
81 Bra
102 Bra
2 Mex
130 US
3 Per
19 DR
7 US
39 DR
47 DR
38 DR
55 DR
5 Per

Figure 3.4

congruence with some sub-clusters in the principal component analyses. Races 38, 39 and 47 showed a tight cluster in both analyses. Races 31, 87, 23 and 15, which formed a tight sub-cluster in cluster B (Figure 3.2) showed 88% similarity in the phenetic analyses. In contrast, race 8 that clustered along with Andean races in cluster C (Figure 3.2) clustered among Middle American races in cluster I (Figure 3.4). Multivariate analyses showed congruence of the 34 races with the *P. vulgaris* gene pools.

DISCUSSION

Phaseolus vulgaris germplasm carrying different combinations of resistance genes from sources in Brazil, the Dominican Republic, Honduras, Mexico and the United States demonstrated a reciprocal influence of variability in *C. lindemuthianum* with the diversity in *P. vulgaris*. The genotypes were grouped by multivariate analyses on the basis of gene pools (Andean and Middle American) and within each gene pool, genotypes were grouped according to their overall resistance levels to *C. lindemuthianum*. Cluster A was the most interesting since it contained genotypes from both gene pools. Among the Andean genotypes were members of the differential series such as MDRK, Perry Marrow and Kaboon, and the cultivar Montcalm, known to carry the same *Co-1* gene as MDRK, and the cultivar Charlevoix, bred for resistance to the Andean race 130 (Andersen et al., 1963). In addition, Isles (Kelly et al., 1994) and Redhawk (Kelly et al., 1997) bred for increased levels of resistance to anthracnose and known to possess the *Co-1* and *Co-2* genes, were present. No information on the other Andean cultivar, Pompadour Checa 50 from the Dominican Republic was available. However, its presence within cluster A

would suggest that it possesses similar high levels of resistance as the other germplasm in cluster A in contrast to the susceptible Andean germplasm in cluster B. The four Middle American cultivars in cluster A included Seafarer, known to possess the Andean *Co-1* gene, Widusa previously classified as being of Andean origin (Sicard et al., 1997), the Mexican cultivars Pinto Villa and Bayo Victoria, reported to carry resistance genes from the Andean parent Canario 101 (Acosta-Gallegos et al. 1995). It would appear that cluster A is comprised of Andean resistance genes regardless of whether they are present in Andean cultivars or have been introgressed into Mesoamerican cultivars. Cluster B comprises highly susceptible Andean genotypes such as Taylor Horticultural and Cardinal from the United States and Cacahuate (cranberry) from Mexico and the United States kidney bean Isabella. Isabella does not possess the *Co-1* gene present in other kidney bean cultivars such as Montcalm, MDRK and Charlevoix (Young and Kelly, 1996). Genetic differences, confirmed from other studies, are supported by the principal component analysis in our study. Middle American genotypes with known sources of resistance, and cultivars bred for resistance, to *C. lindemuthianum* predominated in cluster C. In contrast, the Mesoamerican genotypes in cluster D are older landraces, traditional cultivars, or modern cultivars bred for characteristics other than anthracnose. For instance, a number of cultivars from Honduras such as Tio Canela 75 and MD 3037 were bred primarily for resistance to Bean Golden Mosaic Virus (Rosas et al., 1997) as opposed to the cultivar Catrachita (in cluster C) bred for resistance to *C. lindemuthianum* (Young and Kelly, 1996). Two members of the differential series fell in cluster D, Michelite the universal susceptible cultivar, and Mexico 222 (source of *Co-3* gene) recognized for its limited resistance to *C. lindemuthianum* (Menezes and Dianese, 1988;

Rava et al., 1993; Rodriguez-Guerra, 1991; Restrepo, 1994; Pastor-Corrales et al., 1995). Other cultivars clustering in cluster D include Carioca and Rio Tibagi, the most widely grown cultivars in Brazil (Voysest et al., 1994), a number of Mexican cultivars not recognized as possessing resistance to anthracnose, the susceptible landrace cultivar Zamorano from Honduras and the cultivars Schooner and C-20 from the United States bred for high yield (Kelly et al., 1982).

Phenetic analysis showed both cultivars and races grouped congruently to the *P. vulgaris* gene pools and groups of genotypes with similar overall resistance were observed (Figures. 3.3, 3.4). No grouping of germplasm based on geography alone was observed. Since few sources have been used in breeding for resistance to *C. lindemuthinaum*, groups of genotypes possessing similar resistance genes were observed. For instance, cultivars Charlevoix, Seafarer, Montcalm and Redhawk known to possess the *Co-1* resistance gene, grouped in cluster I. In contrast, cultivars Isabella, Chihuahua 21, Bayomex, Taylor Horticultural and Cardinal with no known resistance genes grouped in a different cluster.

Germplasm with resistance to *C. lindemuthianum* may have increased the genetic diversity among genotypes because genotypes with no resistance exhibited higher similarity than did those genotypes with resistance to the pathogen (Figure 3.4). Large genetic distances were observed among clusters. Clusters II and III showed high overall resistance. In cluster II resistance from Andean sources predominated, despite the gene pool of the genotypes. For instance, the resistance to *C. lindemuthianum* present in the cultivars Seafarer, Pinto Villa and Widusa is from Andean sources despite the fact these

cultivars are from the Middle American gene pool. Cluster III showed the largest genetic dissimilarity in relation to the other clusters.

Congruence among other bean pathogens and host gene pools was reported (Stavely, 1982; Stavely, 1984; Guzman et al., 1995; Maclean et al., 1995; Pastor-Corrales, 1996; Sicard et al., 1997). In all cases, pathogens were divided into two groups corresponding to the Middle American or Andean gene pools. Phenetic analysis indicated that virulence factors might have been selected according to the predominant host gene pool within each country (Figure 3.4). Selection of specific virulence factors in Honduras and the Dominican Republic resulted in the predominance of Middle American and Andean races in each country, respectively (Table 3.2). For instance, races from Honduras overcame no Andean resistance genes present in the differential series, whereas races 38, 39, 47, and 55 from the Dominican Republic overcame all Andean resistance sources present in the differential series. Germplasm grown in Mexico and the United States belongs to both gene pools. Races from these countries overcame resistance sources from both the Andean and Middle American gene pools (Table 3.2). These races were observed in all but cluster IV (Figure 3.4). In contrast, germplasm grown in Brazil belongs mostly to the Middle American gene pool (Voysest et al., 1994). Nevertheless, races carrying virulence factors to both host gene pools have been reported consistently (Meneses and Dianese, 1988; Balardin et al., 1990). For instance, races 31, 55 and 87 overcame Andean resistance sources present in MDRK and Perry Marrow, whereas race 55 also overcame the highly resistant Andean cultivar Kaboon. The principal component analysis grouped the races from Brazil within cluster A (race 1), cluster B (races 31 and 87), and cluster C (races 55, 102 and 130) confirming the pathogen diversity (Figure 3.2).

The Andean races, 15 and 23, consistently clustered among Middle American races in cluster B (Figure 3.2) and cluster I (Figure 3.4). In contrast, race 8, from the Andean region and virulent to the Middle American *Co-2* gene, clustered along with Andean races in cluster C (Figure 3.2) and with the Middle American races in cluster I (Figure 3.4). These results suggest the presence of both Middle American and Andean virulence factors within these races. Similarly, race 31 and 87 virulent to the Andean resistance sources *Co-1* and Perry Marrow, have been consistently reported in Brazil on Middle American hosts (Menezes and Dianese, 1988; Balardin et al., 1990). Nevertheless, these races were grouped along with Andean races 15 and 23 in cluster B (Figure 3.2) and showed high similarity with the same races in cluster I (Figure 3.4). Therefore, categorization of races within a specific reaction group based on origin of isolates and virulence could be biased if broader virulence would result from adaptation of some *C. lindemuthianum* races to both host gene pools. Our virulence data would suggest that the 34 races appear to effectively identify the presence of resistance genes of distinct genetic origin. The clusters, generated as a result of the multivariate analysis, would suggest congruency between the pathogen variability and the host diversity.

Implications on breeding strategy. The large number of virulence factors within the*C. lindemuthianum* population overcame resistance in all genotypes but the cultivar G 2333. The combination of resistance genes *Co-4*^{2}, *Co-5*, and *Co-7* appeared to create greater genetic variability in G 2333 (Young et al., 1997), which remained resistant to all races in our work. Previous work based on virulence analyses of variability in *C. lindemuthianum* showed no clustering of races according to the host gene pools of the

differential cultivars (Balardin et al., 1997). However, the larger set of genotypes in this study appears to be most distinguishing for virulence factors within races of *C. lindemuthianum*. Different genes for virulence and resistance in both Middle America and Andean groups might have been selected as a result of host divergence (Gepts, 1988). Differences among groups of races specialized in a specific host gene pool seem to be derived from the adaptive process of races to hosts. Pyramiding genes based on a knowledge of the complementary effect of large numbers of genes has been suggested as an approach to increase the longevity of resistance under field conditions (Duvick, 1996). For instance, the incorporation of Andean resistance genes in bean breeding populations in Honduras could result in more durable resistance within the country. Similarly, incorporation of Middle American resistance genes in germplasm in the Dominican Republic also would result in more durable resistance. In contrast, the presence of genotypes from both the Andean and Middle American gene pools in a country might have led to the selection of broader virulence in *C. lindemuthinaum* than in those countries where only one or the other gene pools existed. In these countries the use of resistance genes, based on their complementary action to the races currently present, would be necessary to exclude most of the pathogen variability.

LIST OF REFERENCES

Acosta-Gallegos, J. A., Ochoa-Márquez, R., Arrieta-Montiel, M. P., Ibarra-Pérez, F., Ravelero A. P., and Valdez, I. S. 1995. Registration of 'Pinto Villa' common bean. Crop Sci. 35:1211.

Andersen, A. L., Adams, M. W., and Whitford, G. 1963. Charlevoix anthracnose-resistant dark red kidney bean. Mich. Agric. Exp. Stn. Res. Rep. 6:1-3.

Andrus, C. F., and Wade, B.L. 1942. The factorial interpretation of anthracnose resistance in beans. Pages 1-29 in: Tech. Bull. No. 310. USDA. Washington.

Balardin, R. S. and Kelly, J. D. 1997. Re-characterization of *Colletotrichum lindemuthianum* races. Ann. Rep. Bean Improv. Coop. 40:126-127.

Balardin, R.S., Jarosz, A. M., and Kelly, J.D. 1997. Virulence and molecular diversity in *Colletotrichum lindemuthianum* from South, Central and North America. Phytopathology 87:(in press).

Balardin, R. S., Pastor-Corrales, M.A., and Otoya, M.M. 1990. Variabilidade patogênica de *Colletotrichum lindemuthianum* no Estado de Santa Catarina. Fitopatol. Bras. 15:243-245.

Barrus, M. F. 1918. Varietal susceptibility of beans to strains of *Colletotrichum lindemuthianum* (Sacc. & Magn.) B. & C.. Phytopathology 8:589-605.

Blondet, A. 1963. L'anthracnose du haricot: etudé des races physiologiques du *Colletotrichum lindemuthianum*. Ph.D. thèse, Faculté de Sciense, Paris, France.

Burkholder, W. H. 1923. The gamma strain of *Colletotrichum lindemuthianum* (Sacc. et Magn.) B.et C.. Phytopathology 13:316-323.

Casela, C. R., Ferreira, A.S., Zeller, K.A., Levy, M. 1995. Pathotype variation in the sorghum anthracnose fungus: a phylogenetic perspective for resistance breeding. Pages 257-288 in: Disease analysis through genetics and biotechnology. J. F. Leslie and R. S. Frederiksen, eds. Iowa State University Press. Ames, IO.

Dillard, H.R. and Cobb, A.C. 1993. Survival of *Colletotrichum lindemuthianum* in bean debris in New York State. Plant Dis. 77:1233-1238.

Duvick, D.N. 1996. Plant Breeding, an evolutionary concept. Crop Sci. 36:539-548.

Fabre, J. V., Julien, J., Parisot, D., and Dron, M. 1995. Analysis of diverse isolates of *Colletotrichum lindemuthianum* infecting common bean using molecular markers. Mycol. Res. 99:429-435.

Fouilloux, G. 1979. New races of bean anthracnose and consequences on our breeding programs. Pages 221-235 in: Disease of tropical food crops. H. Maraite and J.A. Meyer, eds. Louvain-la-Neuve, Belgium.

Garrido, E. R. 1986. Identificación de razas fisiológicas de *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scrib. en Mexico y busqueda de resistência genética a este hongo. MSc thesis, Institución de Ensenanza y Investigación en Ciencias Agrícolas, Montecillos, Mexico.

Gepts, P. 1988. A Middle American and an Andean common bean gene pool. Pages 375-390 in: Genetic resources of *Phaseolus* beans; their maintenance, domestication, and utilization. P. Gepts, ed. Kluwer. London.

Guzman, P., Donado, M. R., and Galvez, G. E. 1979. Perdidas economicas causadas por la antracnosis del frijol *Phaseolus vulgaris* en Colombia. Turrialba 29:65-67.

Guzman, P., Gilbertson, R.L., Nodari, R., Johnson, W.C., Temple, S.R., Mandala, D., Mkandawire, A.B.C., and Gepts, P. 1995. Characterization of variability in the fungus *Phaeisariopsis griseola* suggests coevolution with the common bean (*Phaseolus vulgaris*). Phytopathology 85:600-607.

Kelly, J. D., Adams, M. W., Saettler, A. W., Hosfield, G. L., and Ghaderi, A. 1984. Registration of C-20 navy bean. Crop Sci. 24:822.

Kelly, J. D., Afanador, L., and Cameron, L.S. 1994. New races of *Colletotrichum lindemuthianum* in Michigan and implications in dry bean resistance breeding. Plant Dis. 78:892-894.

Kelly, J. D., Hosfield, G. L., Varner, G. V., Uebersax, M. A., Long, E. A., and Taylor, J. 1994. Registration of 'Isles' dark red kideny bean. Crop Sci. 34:1407-1408.

Kelly, J. D., Hosfield, G. L., Varner, G. V., Uebersax, M. A., Long, E. A., and Taylor, J. 1997. Registration of 'Redhawk' dark red kidney bean. Crop Sci. 37 (in press).

Kruger, J., Hoffmann, G.M., and Hubbeling, N. 1977. The kappa race of *Colletotrichum lindemuthianum* and sources of resistance to anthracnose in *Phaseolus* beans. Euphytica 26:23-25.

Levy, M., Correa-Victoria, J., Zeigler, R.S., Xu, S., and Hamer, J. 1993. Genetic diversity of the rice blast fungus in a disease nursery in Colombia. Phytopathology 83:1427-1433.

Maclean, D. J., Braithwaite, K.S., Irwin, J.A.G., Manners, J.M., and Groth, J.V. 1995. Random Amplified Polymorphic DNA reveals relationships among diverse genotypes in Australian and American collections of *Uromyces appendiculatus*. Phytopathology 85:757-765.

Mastenbroeck, C. 1960. A breeding program for resistance to anthracnose in dry shell haricot beans, based on a new gene. Euphytica 9:177-184.

Menezes, J. R., and Dianese, J.C. 1988. Race characterization of Brasilian isolates of *Colletotrichum lindemuthianum* and detection of resistance to anthracnose in *Phaseolus vulgaris*. Phytopathology 78:650-655.

Nei, M. 1972. Genetic distance between populations. The American Naturalist 106:283-292.

Pastor-Corrales, M. A. 1991. Estandarización de variedades diferenciales y de designación de razas de *Colletotrichum lindemuthianum*. Phytopathology 81:694 (abstract).

Pastor-Corrales, M. A. 1996. Traditional and molecular confirmation of the coevolution of beans and pathogens in Latin America. Ann. Rep. Bean Improv. Coop. 39:46-47.

Pastor-Corrales, M. A., Erazo, O.A., Estrada, E. I., and Singh, S. P. 1994. Inheritance of anthracnose resistance in common bean accession G 2333. Plant Dis. 78:959-962.

Pastor-Corrales, M. A., Otoya, M.M., and Molina, A. 1995. Resistance to *Colletotrichum lindemuthianum* isolates from Middle America and Andean South America in different common bean races. Plant Dis. 79:63-67.

Pastor-Corrales, M. A., and Tu, J.C. 1989. Anthracnose. Pages 77-104 in: Bean production problems in the tropics. H. F. Schwartz, and Pastor-Corrales, M.A., eds. CIAT. Cali, Colombia.

Rava, C. A., Molina, J., Kauffmann, M., and Briones, I. 1993. Determinación de razas fisiológicas de *Colletotrichum lindemuthianum* en Nicaragua. Fitopatol. Bras. 18:388-391.

Restrepo, S. 1994. DNA polymorphism and virulence variation of *Colletotrichum lindemuthianum* in Colombia. M.Sc. thesis. Universite Paris IV, Paris-Grignon, France.

Rodriguez, R. 1991. Identificación de razas patogenicas de *Colletotrichum lindemuthianum* (Sacc. y Magn.) Scrib. en el estado de Durango mediante un sistema propuesto internacionalmente y respuesta de genótipos de frijol tolerantes a sequia a razas del patógeno. M.Sc., Parasitologia Agricola, Universidad Autonoma Agraria "Antonio Narro", Buenavista, Mexico.

Rosas, J. C., Varela, O. I., and Beaver, J. S. 1997. Registration of 'Tio Canela-75' small red bean (Race Mesoamerica). Crop Sci. 37:1391.

Schwartz, H. F., Pastor-Corrales, M.A., and Singh, S. 1982. New sources of resistance to anthracnose and angular leaf spot of beans (*Phaseolus vulgaris*). Euphytica 31:741-754.

Sicard, D., Michalakis, Y., Dron, M., and Neema, C. 1997. Genetic diversity and pathogenic variation of *Colletotrichum lindemuthianum* in the three centers of diversity of its host, *Phaseolus vulgaris*. Phytopathology, 87:807-813.

Singh, S. P., Gepts, P., and Debouck, D. 1991. Races of common bean (*Phaseolus vulgaris*, Fabaceae). Econ. Bot. 45:379-396.

Stavely, J.R. 1982. The potential for controlling bean rust by host resistance. Ann. Rep. Bean Improv. Coop. 25:28-30.

Stavely, J.R. 1984. Pathogenic specialization in *Uromyces phaseoli* in the United States and rust resistance in beans. Plant Dis. 68:95-99.

Sutton, B.C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. Pages 1-26 in: *Colletotrichum*-Biology, Pathology and Control. J.A. Bailley and M.J. Jeger, eds. C.A.B. International, Wallingford, UK.

Tu, J.C. 1988. Control of bean anthracnose caused by delta and lambda races of *Colletotrichum lindemuthianum* in Canada. Plant Dis. 72:5-7.

Tu, J.C. 1992. *Colletotrichum lindemuthianum* on bean. Population dynamics of the pathogen and breeding for resistance. Pages 203-224 in: *Colletotrichum*-Biology, Pathology and Control. J.A. Bailley and M.J. Jeger, eds. C.A.B. International, Wallingford, UK.

Voysest, O., Valencia, M. C., and Amexquita, M. C. 1994. Genetic divesity among Latin American Andean and Mesoamerican common bean cultivars. Crop. Sci. 34:1100-1110.

Waterhouse, W. L. 1955. Studies of bean anthracnose in Australia. Proc. Linn. Soc. N.S.W. 80:71-83.

Young, R. A., and Kelly, J.D. 1996. Characterization of the genetic resistance to *Colletotrichum lindemuthianum* in common bean differential cultivars. Plant Dis. 80:650-654.

Young, R.A., and Kelly, J.D. 1997. RAPD markers linked to three major anthracnose resistance genes in common bean. Crop Sci. 37:940-946.

Young, R.A., Melotto, M., Nodari, R.O., and Kelly, J.D. 1997. Marker assisted dissection of oligogenic anthracnose resistance in the common bean cultivar, G2333. Theor. Appl. Genet. (in press)

GENERAL CONCLUSIONS

1. Virulence and molecular analysis showed high genetic variability within *Colletotrichum lindemuthianum*. The greatest variability was observed in Central America, decreasing towards North and South America.

2. The characterization of races based on the differential series appear to be limited because of unequal number of genotypes from each *P. vulgaris* gene pool. In addition, presence of genotypes with resistance governed by two or more resistance genes might bias the interpretation of the gene-for-gene interaction in the *C. lindemuthianum* – *P. vulgaris* pathosystem. Taking together these findings support the lack of congruence between populations of *C. lindemuthianum* and the host gene pools.

3. Grouping races based on virulence to resistance sources from a specific host gene pool did not truly represent its virulence. The Andean races 15 and 23, virulent on Andean sources, clustered consistently among Middle American races. In contrast, the Middle American race 31 virulent to hosts from both the Andean and Middle American gene pool grouped along with Andean races and was collected in Brazil only from Middle American hosts. Similarly, the geographic origin of races was not a consistent factor categorizing races. Race 8, virulent to the Middle American *Co-2* gene, was collected in an Andean country and showed higher levels of similarity to Andean than Middle

American races. Combining virulence and molecular data appears to give more consistent support to race categorization into a specific reaction group (i.e. Middle American or Andean) than assigning an origin for individual races based on the host genotype from which the race was initially collected.

4. Parallel evolution among variability in *C. lindemuthianum* and diversity in *Phaseolus* was demonstrated by virulence analysis based on a large set of host genotypes. Pathogen population was structured according to the bean gene pools. Likewise, the gene pool of resistance genes was the factor, which grouped bean genotypes regardless of the gene pool of the genotype, however, grouping of races did not follow a geographic pattern.

5. Molecular analysis based on RAPDs, ITS-RFLP, and sequencing ITS showed no clear congruence among variability in *C. lindemuthianum* and diversity in *Phaseolus*. The polymorphism in the regions of the genome analyzed by these molecular markers did not follow the evolution in *Phaseolus*.

6. Monitoring variability using virulence and molecular methods appears to be the best approach to resolve the population structure of *C. lindemuthianum*. Molecular polymorphism within similar races illustrates the limitations in the race identification based only on virulence to the anthracnose differential cultivars. In addition, poor sampling of isolates of similar virulence phenotype may underestimate the molecular variability within this pathogen. It would appear that variability based on virulence

analysis is limited and that the more diverse molecular variability in *C. lindemuthianum* is a result of sampling a great portion of the pathogen genome.

7. The population structure of *C. lindemuthianum* based on virulence analysis suggests pyramiding resistance genes as an efficient breeding strategy for improving durable resistance in *Phaseolus*. However, variability detected by molecular markers suggested that known resistance genes are insufficient to compensate for the broader variability in *C. lindemuthianum*. This large variability appears to be resulted from such efficient assexual mechanism of variability that only complex gene combinations based on knowledge of gene complementarity would provide germplasm with lont-term resistance to *C. lindemuthianum*.

APPENDIX A

APPENDIX A

Table A.1 Race designation and origin of *C. lindemuthianum* isolates from samples collected on *Phaseolus* hosts from different gene pools in Argentina, Brazil, the Dominican Republic, Honduras, Mexico and the United States

Race[a]	Isolate[b]	Origin[c]	Host [d]	GP[e]
1	Bra 5.1	Brazil	FT 83120 (black)	MA
1	Bra 10.1	Brazil	FT 83120 (black)	MA
1	Bra 15.1	Brazil	FT 83120 (black)	MA
2	Mex 15.1	Mexico	Cacahuate (cranberry)	A
7	Arg 2.1	Argentina	Phaseolus aborigenous [f]	A
7	Mex 46.1	Mexico	Bayomex (yellow)	A
7	US 12.1	United States	Isabella (kidney)	A
7	US 40.1	United States	Cranberry	A
7	US 55.1	United States	CELRK (kidney)	A
7	US 56.1	United States	Chinook (kidney)	A
7	US 57.1	United States	Cranberry	A
7	US 58.1	United States	Chinabean (red)	A
9	Hon 3.1	Honduras	California Small White	MA
9	Hon 9.1	Honduras	Rojito (red)	MA
9	Hon 19.1	Choluteca	Unknown	-
9	Hon 24.1	Honduras	Desarrural (red)	MA
17	Bra 1.1	Brazil	Carioca (carioca)	MA
17	Bra 8.1	Brazil	Carioca (carioca)	MA

Table A1.1 (cont'd)

17	Bra 12.1	Brazil	Turrialba-4 (black)	MA
17	Bra 14.1	Brazil	Turrialba-4 (black)	MA
17	Bra 16.1	Brazil	Carioca (pinto)	MA
19	DR 2.1	Dominican Republic	Pompadour Checa	A
19	Mex 40.1	Mexico	Negro Mexico (black)	MA
55	DR 1.1	Dominican Republic	Pompadour Checa	A
65	Bra 3.1	Brazil	Rio Tibagi (black)	MA
65	Bra 7.1	Brazil	FT 83120 (black)	MA
65	Bra 11.1	Brazil	Macanudo (black)	MA
65	Bra 13.1	Brazil	Rio Tibagi (black)	MA
65	Bra 17.1	Brazil	Rio Tibagi (black)	MA
65	Bra 19.1	Brazil	Rio Tibagi (black)	MA
65	US 1.1	United States	Mayflower (navy)	MA
65	US 2.1	United States	T-39 (black)	MA
65	US 10.1	United States	navy (white)	MA
65	US 13.1	United States	T-39 (black)	MA
65	US 23.1	United States	T-39 (black)	MA
65	US 28.1	United States	Midnight (black)	MA
65	US 30.1	United States	navy	MA
65	US 31.1	United States	Norstar (navy)	MA
65	US 33.1	United States	T-39 (black)	MA
65	US 38.1	United States	T-39 (black)	MA

65	US 43.1	United States	T-39 (black)	MA
65	US 46.1	United States	T-39 (black)	MA
65	US 50.1	United States	T-39 (black)	MA
73	Bra 2.1	Brazil	Rio Tibagi (black)	MA
73	Bra 6.1	Brazil	AN 910342 (black)	MA
73	Bra 9.1	Brazil	FT 83120 (black)	MA
73	Hon 4.1	Honduras	Mexico 309 (black)	MA
73	Hon 5.1	Honduras	Frijol negro (black)	MA
73	Hon 25.1	Honduras	Oriente (red)	MA
73	Hon 26.1	El Paraíso	Oriente (red)	MA
73	Mex 12.1	Mexico	Phaseolus coccineus	-
73	Mex 24.1	Mexico	T 3144-2 (pinto)	MA
73	US 4.1	United States	Aztec (pinto)	MA
73	US 5.1	United States	92T-8056 (small red)	MA
73	US 6.1	United States	92T-3052 (Great Northern)	MA
73	US 7.1	United States	B90202 (black)	MA
73	US 8.1	United States	Blackhawk (black)	MA
73	US 11.1	United States	Aztec (pinto)	MA
73	US 14.1	United States	Sierra (pinto)	MA
73	US 15.1	United States	Alpine (Great Northern)	MA
73	US 16.1	United States	Red Mexican	MA
73	US 17.1	United States	Olathe (pinto)	MA
73	US 18.1	United States	Cahone (pinto)	MA

73	US 19.1	United States	92T-3055 (pinto)	MA
73	US 21.1	United States	92T-3051 (pinto)	MA
73	US 26.1	United States	Avanti (navy)	MA
73	US 29.1	United States	navy	MA
73	US 32.1	United States	Schooner (navy)	MA
73	US 34.1	United States	Huron (navy)	MA
73	US 35.1	United States	Red Mexican	MA
73	US 36.1	United States	T3054-1 (pinto)	MA
73	US 37.1	United States	Red Mexican	MA
73	US 41.1	United States	Avanti (navy)	MA
73	US 42.1	United States	Mayflower (navy)	MA
73	US 44.1	United States	Vista (navy)	MA
73	US 52.1	United States	Mayflower (navy)	MA
73	US 60.1	United States	Blackhawk (black)	MA
73	US 62.1	United States	Blackhawk (black)	MA
73	US 63.1	United States	Mayflower (navy)	MA
73	US 66.1	United States	Blackhawk (black)	MA
73	US 68.1	United States	Vista (navy)	MA
81	Arg 1.1	Argentina	black	MA
89	Bra 18.1	Brazil	FT 85198 (black)	MA
89	Bra 20.1	Brazil	BRA 410 (black)	MA
193	Mex 49.1	Mexico	Bayo Madero	MA
201	Hon 2.1	Hondudras	Unknown	-

209	Mex 45.1	Mexico	Flor de Mayo Bajio	MA
256	Mex 53.1	Mexico	Criollo Bayo (cream)	MA
257	Mex 10.1	Mexico	Puebla 493 (black)	MA
320	Mex 52.1	Mexico	Criollo Bayo (cream)	MA
321	Mex 7.1	Mexico	Pinto	MA
321	Mex 21.1	Mexico	Unknown	-
321	Mex 26.1	Mexico	Negro Puebla (black)	MA
337	Bra. 4.1	Brazil	FT 83120 (black)	MA
357	Mex 25.1	Mexico	FM x BAT (red)	MA
357	Mex 44.1	Mexico	Mantequilla de Calpan	MA
357	Mex 50.1	Mexico	Jamapa (black)	MA
448	Mex 1.1	Durango	Flor de Mayo (pink)	MA
448	Mex 2.1	Durango	Olathe (pinto)	MA
448	Mex 4.1	Durango	Pinto	MA
448	Mex 47.1	Mexico	Puebla 36	MA
448	Mex 55.1	Mexico	Bayito Criollo	MA
449	Mex 3.1	Durango	L 1213-2 (pinto)	MA
449	Mex 6.1	Durango	Line # 213	-
449	Mex 14.1	Mexico	CIAT 2	MA
449	Mex 16.1	Mexico	Azufrado (yellow)	A
449	Mex 18.1	Mexico	Flor de Mayo (pink)	MA
449	Mex 19.1	Mexico	Canario 107 (yellow)	A
449	Mex 20.1	Durango	black	MA

449	Mex 22.1	Mexico	Cacahuate (cranberry)	A
449	Mex 39.1	Durango	Negro (black)	MA
453	Mex 5.1	Durango	Unknown	-
453	Mex 11.1	Mexico	URG 4516	MA
457	Mex 8.1	Mexico	Queretaro 34	MA
457	Mex 17.1	Mexico	URG 3252	MA
457	Mex 23.1	Mexico	black	MA
457	Mex 51.1	Mexico	Garbancillo (brown)	MA
465	Mex 42.1	Mexico	Negro Criollo (black)	MA
469	Mex 38.1	Mexico	Negro (black)	MA
521	Hon 16.1	Honduras	Vaina Blanca	MA
833	Mex 13.1	Mexico	Unknown	-
1033	Hon 15.1	Honduras	Desarrural (red)	MA
1165	Hon 10.1	Honduras	Unknown	-
1344	Mex 43.1	Mexico	Bayo Blanco	MA
1431	Mex 48.1	Mexico	Frijol Negro (black)	MA
1472	Mex 9.1	Mexico	Bayo Madero (cream)	MA
1472	Mex 41.1	Mexico	Azufrado Tapatio	MA
1545	Hon 12.2	Honduras	Frijol Enredadera	MA
1600	Mex 54.1	Mexico	Criollo Negro (black)	MA
1601	Hon 22.1	Honduras	Phaseolus vulgaris (wild)	MA
1673	Hon 1.1	Honduras	Frijol Negro	MA
1673	Hon 13.1	Honduras	Guatemalita	MA

1677	Hon 27.1	Honduras	DOR 304 (red)	MA
1741	Hon 6.1	Honduras	Catrachita (red)	MA
1929	Hon 18.1	Honduras	Unknown	-
1993	Hon 8.1	Honduras	11 - 4	MA
1993	Hon 11.1	Honduras	Vaina Blanca	MA
1993	Hon 20.1	Honduras	Unknown	-
1993	Hon 23.1	Honduras	Yeguare (red)	MA

[a] Race identification according to binary nomenclature system (Pastor-Corrales, 1991)
[b] Identification of isolates in the permanent collection of the Bean Breeding and Genetics Laboratory at the Michigan State University, Crop and Soil Sciences Department considering the country where samples were collected followed by the entry number.
[c] Origin: Arg (Argentina), Bra (Brazil), DR (Dominican Republic), Hon (Honduras), Mex (Mexico), and US (United States).
[d] Host seed class and/or color in parenthesis.
[e] Host Gene Pool: A - Andean; MA - Middle American.
[f] Poroto del zorro (wild Andean).

APPENDIX B

APPENDIX B

Table B 1. Anthracnose differential series and the binary number of each cultivar.

Cultivar	Binary number[a]
Michelite	1
Michigan Dark Red Kidney	2
Perry Marrow	4
Cornell 49242	8
Widusa	16
Kaboon	32
Mexico 222	64
PI 207262	128
TO	256
TU	512
AB 136	1024
G 2333	2048

[a] Binary number: 2^n, being n equivalent to the place of the cultivar within the series. The sum of cultivars with susceptible reaction will give the binary number of one race.
Ex: race 17, virulent on Michelite (1) and Widusa (16).

APPENDIX C

APPENDIX C

Contruction of a SCAR PCR-based marker for differentiation among Andean and Middle American races

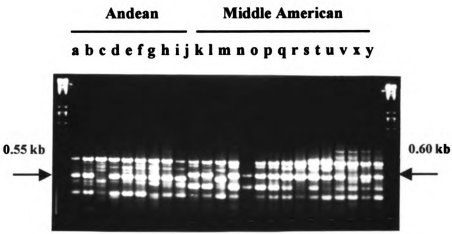

Figure C.1. Randomly amplified polymorphic DNA (RAPD) amplicons obtained with primer 2 of Operon kit G for 24 single-spore isolates of *Colletotrichum lindemuthianum*: a- 2 (Peru), b- 3 (Peru), c- 5 - (Peru), d- 7 (Peru), e- 38 (Dominican Republic), f- 55 (Dominican Republic), g- 15 (Colombia), h- 102 (Brazil), i- 130 (United States), j- 23 (Brazil), k- 87 (Brazil), l- 31 (Brazil), m- 17 (Brazil), n- 65 (Brazil), o- 81 (Brazil), p- 89(Brazil), q- 453 (Mexico), r- 73 (Mexico), s- 449 (Mexico), t- 457 (Mexico), u- 1673 (Honduras), v- 1993 (Honduras), x- 201 (Honduras), y- 2047 (Costa Rica). The polymorphic amplicon indicated by an arrow distinguished among Andean and Middle American races. The construction of a SCAR PCR-based marker was attempted isolating this band from a 0.005% EtBr-stained agarose gel under UV light after electrophoresis. The isolated PCR product was cloned into *Escherichia coli* competent cells using the vector pCR® 2.1 (Invitrogen Co., Carlsbad, CA). The fragment was sequenced using the fluorescent dye dideoxy nucleoside triphosphate terminator method (MSU-DNA Sequencing Facility, East Lansing MI). Sequencing reactions were run on a polyacrylamide gel using the ABI 373A DNA Sequencer. Primers were constructed considering the primer 2 of Operon kit G as starting sequence. No polymorphism was observed using 22-, 24 and 26-base primers.

APPENDIX D

APPENDIX D

Table D.1. Reaction of 62 genotypes of *P. vulgaris* to 20 races of *C. lindemuthianum*.

Germplasm[a]	G P[b]	Races[c]																				RI[d] %
		Arg	Bra							Mex						US						
		81	1	17	31	87	89	337	453	2	257	321	357	449	457	7	23	65	73	102	130	
Carioca	MA	I	I	S	S	I	I	I	I	R	I	I	I	R	R	R	S	S	S	R	R	37
Rio Tibagi	MA	R	S	S	S	R	S	R	I	R	S	R	S	S	-[5]	R	S	S	S	R	R	53
FT 83-120	MA	R	I	I	R	I	R	S	S	R	S	S	S	S	R	S	I	I	S	R	I	21
Macanudo	MA	R	R	I	I	I	R	R	I	R	R	R	R	S	S	R	R	I	R	R	R	74
Danli 46	MA	I	S	S	S	S	R	S	S	I	I	S	S	S	I	I	S	S	R	I	I	26
Desarrural IR	MA	I	I	S	S	S	S	S	I	I	S	I	I	S	I	R	S	S	S	S	I	5
Dorado	MA	I	I	S	S	S	I	S	S	R	S	S	S	S	S	R	S	S	S	R	R	32
Tio Canela 75	MA	I	I	S	S	S	I	S	S	R	S	S	S	S	S	S	S	S	S	R	I	16
Catrachita	MA	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
Yeguare	MA	R	R	R	R	I	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	100
MD 3037	MA	I	I	S	S	S	I	S	S	R	S	S	S	S	S	S	S	S	S	R	R	21
Zamorano	MA	I	R	S	I	S	I	I	S	S	S	S	I	I	I	I	I	S	S	I	I	10
MD 2324	MA	I	R	S	S	S	S	S	I	R	R	I	I	S	S	I	S	S	S	R	R	42
Criollo Negro	MA	R	R	S	R	S	R	I	I	R	S	S	S	S	R	I	R	S	S	I	I	53

Table D.1 (cont'd)

Amarillo 169	MA	I	R	S	S	S	I	R	I	R	S	S	S	R	R	R	S	S	S	R	R	58
Negro Durango	MA	R	I	S	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	I	R	26
Gilguerillo	MA	R	I	S	S	S	S	I	S	R	S	S	S	S	S	R	S	S	S	R	R	58
Puebla 36	MA	R	I	S	R	S	I	I	I	R	S	S	S	S	S	I	S	S	S	I	R	58
Bayo Victoria	MA	R	R	S	R	S	I	I	R	R	R	R	R	R	R	R	S	R	R	R	R	63
Amarillo de Calpan	MA	R	R	S	R	I	R	R	I	R	S	S	S	I	S	R	S	I	I	R	R	68
Durango 32	MA	R	I	S	S	S	S	I	S	R	S	S	S	S	S	R	I	S	S	I	R	42
Azufrado	MA	I	I	S	S	S	S	S	S	S	R	R	R	S	S	R	R	I	S	R	R	42
Flor de Mayo	MA	R	R	I	I	S	I	S	S	I	S	S	S	S	S	I	S	S	R	R	R	32
Pinto Villa	MA	R	R	S	I	S	I	S	R	R	R	R	R	R	R	R	I	R	R	R	R	63
Zacatecas 15	MA	R	R	R	R	R	R	R	S	R	S	S	S	S	S	I	R	R	R	R	R	89
FM M38	MA	R	R	R	R	R	R	R	R	R	S	R	R	I	S	R	R	R	R	R	R	95
Michoacan 8-A	MA	I	I	S	S	S	I	R	S	R	S	S	S	S	R	R	S	S	S	R	R	42
Bayo Berrendo	MA	R	I	S	I	S	I	S	I	R	S	S	S	S	S	R	S	S	S	R	R	21
Negro 150	MA	R	R	R	I	R	R	I	I	R	S	S	S	I	R	I	R	I	R	R	R	79
Seafarer	MA	I	R	R	S	S	R	R	S	R	R	R	R	S	S	I	S	I	R	R	R	37
C-20	MA	R	S	S	I	S	I	S	S	R	I	S	S	S	S	I	I	S	S	R	R	58
Schooner	MA	I	I	R	S	S	S	S	S	R	S	S	S	S	S	I	S	S	R	R	I	32
Newport	MA	R	R	R	I	I	R	R	I	R	R	R	S	S	S	R	I	I	I	R	R	63
Blackhawk	MA	R	R	R	R	R	I	I	R	R	R	R	I	I	S	R	R	I	S	R	R	79

T-39	MA	I	I	S	S	S	S	S	S	R	I	S	S	S	S	I	S	S	S	R	I	26
SEL 1308	MA	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
SEL 1360	MA	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
BAT 93	MA	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
Michelite	MA	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	R	16
Cornell 49242	MA	R	R	R	S	R	S	R	R	R	R	R	R	R	S	R	R	R	S	R	R	68
Widusa	MA	S	R	S	S	S	S	S	R	R	R	R	R	R	R	R	S	R	R	R	R	68
Mexico 222	MA	S	R	R	R	S	S	S	S	R	R	S	S	S	S	R	R	S	S	S	R	84
PI 207262	MA	R	R	R	R	R	R	R	S	R	R	R	R	S	S	R	R	R	R	R	S	100
TO	MA	R	R	R	R	R	R	S	S	R	S	S	S	S	S	R	R	R	R	R	R	100
TU	MA	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
AB 136	MA	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
G 2333	MA	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
Pompadour Checa 50	A	I	R	R	R	I	R	I	R	-[5]	R	R	S	R	R	I	R	I	R	R	I	61
Chihuahua 21	A	R	R	S	I	S	I	S	I	S	S	S	S	I	I	S	I	S	S	S	S	10
Bayomex	A	R	R	S	S	S	I	S	S	S	S	S	S	S	S	S	I	S	S	S	S	10
Cacahuate 72	A	R	R	S	S	S	I	S	R	S	S	S	S	R	R	S	S	S	S	S	S	16
Montcalm	A	R	R	R	S	S	R	R	S	I	R	R	S	S	S	S	S	R	R	I	S	31
Ruddy	A	R	I	S	S	S	I	S	S	R	S	I	S	R	R	R	S	S	S	R	R	42
Isabella	A	I	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0
Isles	A	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	63

Red Hawk	A	R	R	R	S	S	R	I	S	R	R	R	S	I	I	S	S	R	R	R	R	42
Charlevoix	A	R	R	R	S	S	R	I	S	S	I	R	S	R	R	S	S	R	R	S	S	42
Taylor Horticultural	A	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S	5
Cardinal	A	R	S	S	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	5
MDRK	A	R	R	R	S	S	R	R	R	S	R	R	R	R	R	S	S	R	R	S	S	42
Perry Marrow	A	R	R	R	S	S	R	R	S	R	R	R	S	R	R	S	S	R	R	S	R	47
Kaboon	A	R	R	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	79

[1] Seed provided by: Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), Brazil/RS; Escuela Agricola Panamericana Zamorano, Honduras; National Research Institute for Forestry and Agriculture (INIFAP), Mexico; Centro Internacional de Agricultura Tropical (CIAT), Colombia; Bean Breeding and Genetics Program (Michigan State University), United States.

[2] A – Andean gene pool; MA – Middle American gene pool.

[3] Designation of races based on the binary nomenclature system (Pastor-Corrales, 1991). Races previously designated by Greek letters: 17 - alpha (α), 55 - lambda (λ), 65 - epsilon (ε), 81 - mu (μ), 89 - alpha-Brazil (α), 453 - zeta (ζ) (Balardin and Kelly, 1997). Origin of races: Arg (Argentina), Bra (Brazil), Mex (Mexico), and US (United States).

[4] Resistance index = (total no. of resistant reactions - R) / 20, being 20 the total number of races inoculated on each genotype.

[5] Data missing.

APPENDIX E

APPENDIX E

Table E.1. Reaction of 62 genotypes of *P. vulgaris* to 14 races of *C. lindemuthianum*.

Germplasm[a]	G P[b]	Races[c]														RI[d] %
		Clb	CR	Hon				Per			DR					
		15	2047	9	521	1673	1993	3	5	8	19	38	39	47	55	
Carioca	MA	I	I	I	R	R	I	I	I	I	R	R	R	R	R	40
Rio Tibagi	MA	S	I	I	R	R	S	R	R	I	R	R	R	R	R	47
FT 83-120	MA	S	I	I	R	R	S	I	S	S	I	S	S	S	R	33
Macanudo	MA	R	I	R	R	I	S	R	R	I	R	R	I	R	R	53
Danli 46	MA	I	I	I	S	I	S	R	R	R	R	I	S	S	I	7
Desarrural IR	MA	I	S	S	S	S	S	S	S	I	I	I	S	S	I	0
Dorado	MA	S	I	S	I	R	S	R	R	I	R	I	S	S	R	20
Tio Canela 75	MA	I	I	I	R	I	S	R	I	S	S	S	S	S	R	14
Catrachita	MA	R	I	R	R	R	S	R	R	R	R	R	R	R	R	87
Yeguare	MA	R	I	R	S	I	S	R	R	R	R	R	R	R	R	54
MD 3037	MA	I	I	S	R	R	S	R	R	I	I	S	I	S	R	27
Zamorano	MA	S	I	S	S	S	S	I	R	I	I	S	I	S	S	0
MD 2324	MA	S	S	I	R	R	I	R	R	R	R	I	I	R	R	34
Criollo Negro	MA	I	S	I	S	S	S	R	R	I	R	R	S	R	I	14

Table E.1 (cont'd)

Amarillo 169	MA	I	S	I	S	S	S	R	R	R	R	R	R	R	34
Negro Durango	MA	S	S	S	S	I	S	I	I	I	S	R	I	R	I	7
Gilguerillo	MA	R	S	I	S	S	S	R	R	R	R	R	R	I	R	14
Puebla 36	MA	I	S	I	S	S	S	R	R	R	R	R	R	R	R	7
Bayo Victoria	MA	I	I	R	R	R	R	R	R	R	S	I	S	I	R	73
Amarillo de Calpan	MA	I	S	R	I	I	I	R	R	R	R	R	I	R	R	27
Durango 32	MA	I	S	S	S	I	S	I	I	R	R	R	R	R	I	7
Azufrado	MA	S	S	S	R	I	S	I	R	S	R	R	R	R	R	40
Flor de Mayo	MA	R	S	S	S	I	S	R	I	I	I	S	S	I	R	14
Pinto Villa	MA	R	S	R	R	R	R	R	R	R	I	S	I	I	R	73
Zacatecas 15	MA	R	S	R	R	I	S	I	R	R	R	R	R	R	R	40
FM M38	MA	S	S	R	R	R	S	R	R	R	R	R	R	R	R	67
Michoacan 8-A	MA	I	S	S	S	S	S	I	I	R	R	R	R	R	R	27
Bayo Berrendo	MA	I	S	S	I	S	I	S	R	S	I	S	S	I	I	14
Negro 150	MA	I	I	R	R	I	S	R	R	R	R	R	R	R	R	40
Seafarer	MA	S	S	R	R	R	S	R	R	I	I	I	I	I	I	60
C-20	MA	R	S	S	R	I	S	R	R	R	R	R	R	R	R	20
Schooner	MA	I	S	S	R	I	S	R	R	I	I	S	S	S	R	14
Newport	MA	I	S	R	R	R	S	R	R	R	R	I	I	R	R	54
Blackhawk	MA	I	S	S	R	R	S	R	R	R	R	R	R	R	R	54

T-39	MA	S	S	S	R	R	S	S	S	I	R	R	R	S	R	20
SEL 1308	MA	R	R	R	R	R	I	R	R	R	R	R	R	R	R	93
SEL 1360	MA	R	R	R	R	R	I	R	R	R	R	R	R	R	R	93
BAT 93	MA	R	S	R	S	I	S	R	R	R	R	R	R	R	R	67
Michelite	MA	S	S	S	S	S	S	S	S	R	S	R	S	S	S	14
Cornell 49242	MA	S	S	S	S	S	S	R	R	S	R	R	R	S	R	60
Widusa	MA	R	S	R	R	R	R	R	R	R	S	R	R	R	S	73
Mexico 222	MA	R	S	R	R	R	S	R	R	R	R	R	R	R	R	27
PI 207262	MA	R	S	R	R	S	S	R	R	R	R	R	R	R	R	53
TO	MA	R	S	R	R	R	S	R	R	R	R	R	R	R	R	40
TU	MA	R	S	R	S	S	S	R	R	R	R	R	R	R	R	74
AB 136	MA	R	S	R	R	S	S	R	R	R	R	R	R	R	R	80
G 2333	MA	R	R	R	R	R	R	R	R	R	R	R	R	R	R	100
Pompadour Checa 50	A	R	S	R	R	R	R	R	R	R	R	S	S	S	S	67
Chihuahua 21	A	S	I	S	S	S	S	I	I	S	I	S	I	S	S	0
Bayomex	A	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0
Cacahuate 72	A	S	S	S	S	S	S	S	R	S	S	S	S	S	S	20
Montcalm	A	S	S	R	R	R	S	I	S	S	I	S	I	S	I	40
Ruddy	A	S	S	R	R	R	R	R	R	R	R	S	S	S	S	53
Isabella	A	S	I	S	S	S	S	S	S	S	S	S	S	S	I	0
Isles	A	S	S	R	R	R	R	I	R	R	R	R	S	S	I	87

Red Hawk	A	S	S	R	R	R	S	I	S	S	R	S	S	S	I	47
Charlevoix	A	S	S	R	R	R	I	S	R	R	S	S	S	S	S	40
Taylor Horticultural	A	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0
Cardinal	A	S	S	S	S	S	S	I	S	S	S	S	S	S	S	0
MDRK	A	S	S	R	R	R	R	S	R	R	S	S	S	S	S	73
Perry Marrow	A	S	S	R	R	R	R	R	S	R	R	S	S	S	S	67
Kaboon	A	R	R	R	R	R	R	R	R	R	R	S	S	S	S	80

[a] Seed provided by: Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), Brazil/RS; Escuela Agricola Panamericana Zamorano, Honduras; National Research Institute for Forestry and Agriculture (INIFAP), Mexico; Centro Internacional de Agricultura Tropical (CIAT), Colombia; Bean Breeding and Genetics Program (Michigan State University), United States.

[b] A – Andean gene pool; MA – Middle American gene pool.

[c] Designation of races based on the binary nomenclature system (Pastor-Corrales, 1991), races previously designated by Greek letters: 17 - alpha (α), 55 - lambda (λ), 65 - epsilon (ε), 81 - mu (μ), 89 - alpha-Brazil (α), 453 - zeta (ζ) (Balardin and Kelly, 1997). Origin of races: Clb (Colombia), CR (Costa Rica), Hon (Honduras), Per (Peru), and DR (Dominican Republic).

[d] Resistance index = (total no. of resistant reactions - R) / 14, being 14 the total number of races inoculated on each genotype.