Morphologic, Molecular, and Pathogenic Characterization of *Diaporthe phaseolorum* Variability in the Core Soybean-Producing Area of Argentina

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ABSTRACT

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Isolates of the *Diaporthe/Phomopsis* (D/P) complex were collected in the main soybean producing area of Argentina during the 1996–97, 1997–98, and 1998–99 growing seasons. Twenty-three morphologic characters related to type of colonies, stroma, pycnidia and conidia, presence of perithecia, and asci length were studied by principal component analysis (PCA). Genomic DNA were analyzed by the random amplified polymorphic DNA (RAPD) technique. From both studies, 18 isolates were identified as D/P complex and grouped in four major taxa: (i) *Diaporthe phaseolorum* var. *meridionalis*, (ii) *D. phaseolorum* var. *caulivora*, (iii) *D. phaseolorum* var. *sojae*, and (iv) *Phomopsis longicolla*. In addition to distinguishing interspecific and intraspecific variability, molecular markers allowed the detection of differences among isolates within the same variety. Pathogenicity was assayed in the greenhouse, by the toothpick method, inoculating the D/P isolates to soybean genotypes carrying different resistance genes (*Rdc1*, *Rdc2*, *Rdc3*, and *Rdc4*) against soybean stem canker (SSC). Pathogenic analysis distinguished two main groups: (i) the SSC-producing isolates, including *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora*, and (ii) the non-SSC-producing

isolates, including *D. phaseolorum* var. *sojae* and *P. longicolla*. Cultivar RA-702 (susceptible control) was compatible with both *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora* isolates; meanwhile, Tracy-M (*Rdc1* and *Rdc2* genes) was incompatible with *D. phaseolorum* var. *meridionalis* but compatible with *D. phaseolorum* var. *caulivora* isolates. The fact that *Rdc1* and *Rdc2* together (as in Tracy-M) confer an almost immune reaction to all assayed isolates of *D. phaseolorum* var. *meridionalis* but were ineffective against the *D. phaseolorum* var. *caulivora* isolates evaluated suggests that the virulence or avirulence genes in *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora* are different. Moreover, physiological races of *D. phaseolorum* var. *meridionalis* were detected by using differential soybean genotypes carrying distinct single *Rdc* genes. As far as we know, this is the first report on the existence of physiological races of *D. phaseolorum* var. *meridionalis* in South America. Selective pressure due to deployment of resistant host cultivars may have changed the frequency of the virulence or avirulence genes within the population of *D. phaseolorum* var. *meridionalis*. On the whole, our results show that pathogenic variability of *D. phaseolorum* in the core soybean-producing area of Argentina is higher than previously recognized.

Additional keywords: *Glycine max*, plant–fungus interaction, pod and stem blight, seed decay, soybean stem canker.

The *Diaporthe/Phomopsis* complex constitutes an important pathogenic group of soybean with ample genetic diversity (22). This complex is composed by three varieties of *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., anamorph *Phomopsis phaseoli* (Desmaz.) Sacc.: *D. phaseolorum* var. *meridionalis* Fernández, and *D. phaseolorum* var. *caulivora* K. L. Athow and R. M. Caldwell, both causal agents of soybean stem canker (SSC), and *D. phaseolorum* var. *sojae* (S. G. Lehman) Wehmeyer, causal agent of pod and stem blight; and by *Phomopsis longicolla* T. W. Hobbs (teleomorph unknown), primary agent of seed decay (8,35,38). *Phomopsis* spp. are recurrent pathogens affecting seed quality and germination (4,26,37). The damage level depends of the environmental conditions (21,41) and infected seed are the main source of primary infection in plots free of this disease (2,32).

In the 2001–02 growing season, Argentina produced 28.4 million tons of soybean. Approximately 90% was produced in the northern pampean subregion, between 30 and 36° south latitude, with Santa Fe Province accounting for over 40% of the total production of the country (34). *Diaporthe* and *Phomopsis* spp. frequently are found in the core soybean-producing area of Argentina, infecting seed mainly at physiological maturity and causing major reductions in seed germination (25,26). SSC, caused by *D. phaseolorum* var. *meridionalis*, first was detected in southern Santa Fe during the 1992–93 growing season (33). The disease caused severe damage in this area, as well as in northwest Argentina, in the 1996–97 growing season, mainly due to the wide diffusion of susceptible cultivars, high inoculum, and predisposing weather conditions (24,47). We reported pathogenic variability among *D. phaseolorum* var. *meridionalis* isolates collected in different locations of the Santa Fe Province, suggesting that genetic diversity is present in local *D. phaseolorum* var. *meridionalis* populations (30). Variability in virulence among isolates of *D. phaseolorum* was previously reported (11,13,14,44). This is not surprising, because the infective form of this fungus in the field is sexual, allowing genetic recombination (39). Recently, we reported for the first time the presence of *D. phaseolorum* var. *caulivora* in Argentina and all South America (28). The isolates cha-

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racterized as *D. phaseolorum* var. *caulivora* were collected in the same geographical area where *D. phaseolorum* var. *meridionalis* was previously identified (28). The coexistence of both varieties of *D. phaseolorum* (i.e., *meridionalis* and *caulivora*) in the core soybean-producing area of Argentina increased the genetic diversity of the pathogen. This potentially could be a major threat when a common source of resistance has been incorporated in widely used cultivars (51). Resistance to SSC was reported to be controlled by at least four major, dominant, nonallelic genes: *Rdc1* and *Rdc2*, present in cv. Tracy-M (17), *Rdc3* in cv. Crockett, and *Rdc4* in cv. Dowling (5). The *Rdc4* gene also was identified in cv. Hutcheson (43). Initially, it was considered that any individual gene would confer complete resistance in the field, but it also was suggested that two genes might provide wider protection than either gene alone (18). However, there is no evidence to indicate additive gene action in SSC resistance (9). On the other hand, there are reports of pathogenic trials in which cultivars resistant to southern stem canker isolates were susceptible to northern stem canker isolates (11,14,15). However, it is not clear at present if the known *Rdc* genes will confer the same degree of resistance to isolates of both varieties of *D. phaseolorum* causing SSC, as well as to distinct isolates within the same variety. The situation prompted the necessity to further characterize pathogenic variability within *D. phaseolorum* in the core soybean-producing area of Argentina as a first step in developing effective strategies for the incorporation of SSC resistance into local commercial cultivars.

Grouping different taxa of the *Diaporthe/Phomopsis* complex has been difficult. Morphologic characteristics have been used to differentiate them, as well as the disease they cause on soybean. However, morphologic characteristics are often too variable to establish appropriate taxonomic ranks (51). The use of molecular analysis is a powerful tool to refine morphologic characterization of genetic variability within this complex, helping to identify closely related isolates difficult to separate phenotypically. Specifically, random amplified polymorphic DNA (RAPD) has been successfully applied to the differentiation of *D. phaseolorum* var. *meridionalis* as a variety of *D. phaseolorum* from soybean (8), and internal transcribed spacers (ITS) of ribosomal DNA to distinguish soybean isolates of *D. phaseolorum* and *P. longicolla* from other soybean pathogens (50), and to infer the phylogeny of the *Diaporthe/Phomopsis* complex on soybean (51).

The objectives of this work were to (i) characterize morphologically and molecularly the different taxa of the *Diaporthe/Phomopsis* complex present in the core soybean-producing area of Argentina and (ii) evaluate the pathogenic variability of local *D. phaseolorum* isolates when inoculated in differential soybean genotypes carrying distinct major genes of resistance to SSC.

MATERIALS AND METHODS

Soybean plants showing typical symptoms of stem canker, pod and stem blight, and seed decay caused by the *Diaporthe/Phomopsis* complex were collected during the 1996–97, 1997–98, and 1998–99 growing seasons, from 14 localities of the Santa Fe Province (between latitude 29 and 34°S) and 1 locality of the Chaco Province (latitude 27°30'S), Argentina (Fig. 1). The Santa Fe isolates were from fields under continued agriculture, where the prevalent crop sequence was: soybean monoculture, wheat and soybean, or corn–soybean–wheat and soybean, under a notillage cropping system. The Chaco isolate was from a field recently dismantled and subsequently cultivated with a sorghum–soybean–soybean sequence, under a notillage cropping system. Identified isolates of *D. phaseolorum* var. *meridionalis* from Brazil, Paraguay, and the United States; *D. phaseolorum* var. *caulivora* and *P. longicolla* from the United States; and *D. phaseolorum* var. *sojae* from Argentina were used as controls (Table 1).

Morphologic characterization. Pathogens were cultured on acidified potato glucose agar (APGA) with 0.2% lactic acid, and

incubated in darkness at $25 \pm 1^\circ\text{C}$ (except as noted below). Cultures were maintained at room temperature (35 to 45 days) to induce fruiting bodies, and then stored at 4°C . SSC isolates were also grown on autoclaved soybean stems on APGA in order to induce fruiting bodies (27,51). Isolates were characterized by the morphology of their colonies, pycnidia, perithecia, α - or β -conidia, asci length, and bicellular and biguttulate ascospores (8,29) and deposited in the Centro de Referencia en Micología (CEREMIC) de la Facultad de Ciencias Bioquímicas y Farmacéuticas (Universidad Nacional de Rosario). All isolates, except DPMNa and DPMNb, were characterized by the in vitro culture appearance of colonies and, when produced, by their fruiting bodies (Table 1). Twenty-three morphologic characteristics related to colony morphology and color, presence and shape of stroma, presence and type of pycnidia and conidia, presence and type of perithecia, and length of ascus and ascospores were compared (Table 2). A stereoscopic microscope ($\times 40$) was used to characterize the colonies and the fruiting bodies. Quantitative measures of the length of asci and ascospores were made, on fucsin-stained preparations, by using a light microscope ($\times 1,000$) equipped with a micrometer ocular ($n = 50$). To group morphologically the different isolates, a principal component analysis (PCA; PADN Systems) was performed. Seventeen isolates were included in the PCA (Table 1) and 23 morphologic characteristics were used to differentiate them (Table 2). Each morphologic characteristic was measured as present or absent and considered significant when their coefficients of correlation were ≥ 0.60 ($P \leq 0.01$, 15 degrees of freedom) (10).

RAPD analysis. Isolates were grown in petri dishes containing APGA medium. After 5 days, the mycelium was harvested and

Fig. 1. Map of Santa Fe Province, Argentina, showing the localities where samples of the *Diaporthe/Phomopsis* isolates were collected.

total DNA was extracted according to the method of Dellaporta et al. (6) with slight modifications. Mycelia were collected with a scalpel (50 to 100 mg of fresh tissue) in 1.5-ml centrifuge tubes, combined with 700 µl of extraction buffer (100 mM Tris HCl, 50 mM EDTA, 500 mM NaCl, 7 mM β-mercaptoethanol, and 1% sodium dodecyl sulfate, pH 8.0) and placed at -80°C for 30 min. Then, samples were incubated at 65°C for 10 min. After that, 250 µl of 5 M potassium acetate was added, and the sample was mixed and placed on ice for 20 min. The mixture was centrifuged at 12,000 rpm for 20 min at 4°C. Supernatants were transferred to new 1.5-ml tubes. One volume of 2-propanol was added, and samples were mixed by gentle inversion and incubated at room temperature for 10 min. Tubes were centrifuged at 12,000 rpm for 15 min at room temperature. Supernatants were discarded and DNA pellets were rinsed twice with 70% ethanol, dried, and dissolved in a proper volume of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8).

RAPD analysis was carried out after a selection of suitable arbitrary decamer primers (Operon Technologies, Valencia, CA). A preliminary screening of 11 primers from kit AA provided 5 primers which yielded amplification products. Only two primers yielded reproducible band patterns: primer OP-AA01 (5'-AGACGGCTCC-3') and primer OP-AA06 (5'-GTGGGTGCCA-3'). The reaction mixture (25 µl) contained 100 µM each dNTP; 2.5 µl of 10× Stoffel buffer (100 mM Tris-HCl, 100 mM KCl, pH 8.3); 4 mM MgCl₂; 0.2 µM primer; 2 units of AmpliTaq DNA Polymerase Stoffel Fragment (Perkin-Elmer, Alameda, CA); and 1 µl of a 1:500 dilution of each DNA. Amplification was performed in a PTC-100 thermal cycler (MJ Research Inc., Waltham, MA) with a preliminary step of 3 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; and a final step of 7 min at 72°C. Samples of 10 µl of polymerase chain reaction (PCR) products were analyzed on 6% denaturing polyacrylamide gels in TBE buffer running at 60W for 3 h. Amplification patterns were

visualized by silver stain (Silver Sequence DNA Sequencing System; Promega Corp., Madison, WI).

Each RAPD band was scored as either present or absent across all fungal genotypes to create a binary basic data matrix (BDM). The dendrogram was constructed by unweighted pair group method with arithmetic means cluster analysis using the Jaccard association coefficient (40) with the SimQual program of NTSYSpc (version 1.8; Exeter Software, Setauket, NY). The cophenetic matrix was calculated and normalized by the Mantel correspondence test.

Pathogenicity trials. Soybean genotypes and resistance genes (*Rdc*) tested were Tracy-M (*Rdc1* and *Rdc2*), line T-1 (homozygous for *Rdc1*), line T-2 (homozygous for *Rdc2*), Crockett (homozygous for *Rdc3*), Dowling (homozygous for *Rdc4*), Hutcheson (homozygous for *Rdc4*), and RA 702 (susceptible control). T-1 and T-2 were F₄-derived lines from a cross between Tracy-M and J77-339, obtained by Kilen and Hartwig (17). Hypocotyls of seedlings at the fully expanded trifoliate leaf stage (12 to 14 days old) were inoculated by the toothpick method (12) in the greenhouse during 1998 and 1999. Each plant genotype was individually inoculated with each fungus isolate. Seedlings either not wounded or punctured with sterile toothpicks and covered with vaseline served as noninoculated controls. Seedlings were kept with high relative humidity (90 to 100%) during the first 72 h after inoculation (29). The result of the plant-fungus interactions was measured as percentage of dead plants (%DP). For the calculation of %DP, a value of 1 was assigned to each dead plant and a value of 0.5 to each plant displaying stem canker symptoms (49). The %DP was measured 30 days after inoculation by computing the proportion of plants that were either dead or displayed SSC symptoms out of the total number of plant assayed. The evaluation period was determined according to the results of a previous study of disease progress curves (29). SSC-producing isolates were re-isolated from the first internode above the inocu-

TABLE 1. Isolates of *Diaporthe phaseolorum* var. *meridionalis*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *sojae*, *Phomopsis longicolla*, and *Diaporthe* spp. used for morphological observations, polymerase chain reaction-random amplified polymorphic DNA (PCR-RAPD), and principal component analysis (PCA)

Isolate code ^a	Species or variety	Geographic location	Collector
Controls			
CE118(2001)**	<i>D. phaseolorum</i> var. <i>sojae</i>	Bigand, Santa Fe (SF), Argentina	R. N. Pioli
CE114(2001)**	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Brazil	M. A. Rott Oliveira
DPMNa****	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Brazil	P. Grijalba
DPMNb****	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Paraguay	P. Grijalba
DPM S(TC)5*	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Illinois, United States	G. L. Hartman
DPCB(IT)*	<i>D. phaseolorum</i> var. <i>caulivora</i>	Illinois, United States	G. L. Hartman
DPC713(IT)*	<i>D. phaseolorum</i> var. <i>caulivora</i>	Illinois, United States	G. L. Hartman
PL99.11.1**	<i>P. longicolla</i>	Illinois, United States	G. L. Hartman
Evaluated			
CE107(2001)**	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Malabrigo, SF, Argentina	R. N. Pioli
CE108(2001)**	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Los Molinos, SF, Argentina	R. N. Pioli
CE109(2001)**	<i>D. phaseolorum</i> var. <i>meridionalis</i>	San Justo, SF, Argentina	R. N. Pioli
CE110(2001)**	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Oliveros, SF, Argentina	R. N. Pioli
CE111(2001)**	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Barrancas, SF, Argentina	R. N. Pioli
CE112(2001)**	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Clarcke, SF, Argentina	R. N. Pioli
CE113(2001)**	<i>D. phaseolorum</i> var. <i>meridionalis</i>	Lucio V. López, SF, Argentina	R. N. Pioli
DPC5a**	<i>D. phaseolorum</i> var. <i>caulivora</i> ^b	Clarcke, SF, Argentina	R. N. Pioli
CE115(2001)**	<i>D. phaseolorum</i> var. <i>caulivora</i> ^b	Clarcke, SF, Argentina	R. N. Pioli
CE116(2001)**	<i>D. phaseolorum</i> var. <i>caulivora</i>	Oliveros, SF, Argentina	R. N. Pioli
DPS99a**	<i>D. phaseolorum</i> var. <i>sojae</i>	Cda. de Gómez, SF, Argentina	R. N. Pioli
CE119(2001)**	<i>D. phaseolorum</i> var. <i>sojae</i>	Zavalla, SF, Argentina	R. N. Pioli
CE120(2001)*	<i>D. phaseolorum</i> var. <i>sojae</i>	Charata, Chaco, Argentina	R. N. Pioli
PI5818*	<i>Diaporthe</i> spp. ^c	Totoras, SF, Argentina	R. N. Pioli
DP4***	<i>Diaporthe</i> spp.	Cnel. Bogado, SF, Argentina	R. N. Pioli
PLCLAS 99a*	<i>P. longicolla</i>	Classon, SF, Argentina	R. N. Pioli
PLCLAS 99b**	<i>P. longicolla</i>	Classon, SF, Argentina	R. N. Pioli
CE117(2001)**	<i>P. longicolla</i>	Totoras, SF, Argentina	R. N. Pioli

^a * = isolates described morphologically and included in the PCR-RAPD analysis, ** = isolates described morphologically and included in the PCA and the PCR-RAPD analysis, *** = isolate described morphologically only, and **** = isolates included in the PCR-RAPD analysis only.

^b Isolates obtained from infected seed.

^c Nonreproductive body producing isolate.

lation point of five randomly chosen symptomatic plants. The identity of re-isolated pathogens was checked further by the induction of sexual and asexual fructifications. In control plants, no symptoms were detected and no mycelia growth occurred from stem portions plated on APGA. In all, 54, 36, and 54 seedlings were used in the first, second, and third experiment, respectively. Interaction between each plant genotype with each fungus isolate was evaluated at least twice in different experiments.

Yorinori (49) proposed five categories for classifying the soybean genotype reaction when inoculated with stem canker-producing isolates under greenhouse conditions. In his scale, a plant genotype was considered resistant when it showed 0 to 25% DP. We used a more stringent level to consider a plant–fungus interaction as incompatible (i.e., plant is resistant). In our scale, incompatible interactions were restricted to less than 15% DP. We also simplified the compatibility scale to four categories of plant–fungus interaction: (i) incompatible (0 to 14.9%), (ii) compatible (15 to 49.9%), (iii) very compatible (50 to 84.9%), and (iv) highly compatible (85 to 100%). Isolates were clustered, based on the category of their interaction with soybean genotypes carrying different *Rdc* genes, by the Ward's Method PC-ORD (version 4; (MjM Software Design, Gleneden Beach, OR) using Euclidean distance as a measure of similarity among them.

RESULTS

Morphologic and in vitro culture characteristics. According to their morphologic characteristics, the 18 local isolates assayed were identified and grouped into the following taxa: *D. phaseolorum* var. *meridionalis*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *sojae*, *Diaporthe* spp., and *P. longicolla* (Table 1).

D. phaseolorum var. *meridionalis* isolates CE107, CE108, CE109, CE110, CE111, CE112, and CE113 showed tufted my-

celium and white colonies that became tanned with age. The underside of a culture was generally light brown to tan. The intensity of color was related to the abundance of hyphae with thick and dark cell walls. Colony development was sustained between 25 and 28°C. Defined stroma were infrequent, but older cultures of the isolate CE108 showed small (1 to 4 mm), circular dark stroma, scattered in the medium. All isolates produced mutic pycnidia with α -conidia after 25 to 30 days in culture. After 35 days in culture, solitary perithecia with long beaks were observed spread on the mycelia and on soybean stem segments artificially inoculated and incubated on APGA medium. The ascus mean length was 36.7 ± 4.7 μ m. The length and width of bicellular biguttulate ascospores were 10.2 ± 1 and 3 ± 0.13 μ m, respectively (Table 2).

D. phaseolorum var. *caulivora* isolates DPC5a and CE115 were obtained from seed and produced SSC symptoms when inoculated on soybean seedlings, and CE116 was obtained from the stem of a field-grown soybean plant exhibiting SSC symptoms (Table 1). Cultural characteristics of these three isolates were morphologically distinct from *D. phaseolorum* var. *meridionalis*, producing white-yellow colonies with compact, shrunken mycelia that changed to ochre and fluffy with age. The underside of the culture was occasionally light ochre to tan. Stromata were infrequent and pycnidia were not produced. The mycelium grew slowly in temperatures higher than 25°C. After 35 to 40 days in culture, perithecia with long beaks were produced. Isolate CE116 formed perithecia in valsoid clusters; meanwhile, isolates DPC5a and CE115 formed both solitary and clustered perithecia. Additionally, ascus mean length of CE116 was 26.9 ± 2.5 μ m, and the mean length and width of its bicellular biguttulate ascospores were 8.3 ± 0.6 and 2.6 ± 0.1 μ m, respectively. However, DPC5a and CE115 showed ascus mean length of 34.0 ± 2.0 m and the length and width of their bicellular biguttulate ascospores were 9.6 ± 0.4 and 3.0 ± 0.13 μ m, respectively.

TABLE 2. Morphological characteristics used to discriminate among isolates of the *Diaporthe/Phomopsis* complex by principal components analysis^a

Species or variety	Morphological characteristics	Principal component, correlation coefficients ^b		
		PC1	PC2	PC3
Colony morphology				
DPC	Compact	0.72**	0.58	...
DPM & DPS	Tufted, rope-like mycelia	-0.66**	-0.73**	...
DPC	Shrunken to fluffy mycelia	0.66**	0.73**	...
Colony color				
DPM	White to tan	0.42	-0.73**	...
DPC	White-yellow to ochre	0.72**	0.58	...
DPS & PL	White, gray, yellow-greenish	-0.91**	0.37	...
Stroma				
DPM	Undefined stroma	0.37	-0.91**	...
DPM & DPC	Infrequent	0.72**	0.58	...
DPS	Frequent and scattered	-0.62**	0.24	0.73**
DPS & PL	Frequent and extended	-0.91**	0.37	...
Pycnidia and conidia				
DPM & DPS	Mutic pycnidia	0.33	-0.49	0.80**
PL	Pycnidia with long beaks	-0.52	0.23	-0.72**
DPM & PL	α -conidia	0.41	-0.50	-0.50
DPS	α - and β -conidia	-0.62**	0.24	0.73**
Perithecia				
DPM & DPC	Perithecia development	0.91**	-0.37	...
DPM	Solitary perithecia	0.76**	-0.60**	...
DPC	Clustered perithecia	0.66**	0.73**	...
Ascus length				
DPM	36.7 ± 4.7 μ m	0.37	-0.91**	...
DPC (seed)	34.0 ± 2.0 μ m	0.59	0.49	...
DPC	26.9 ± 2.5 μ m	0.26	0.51	...
Ascospores length				
DPM	10.2 ± 1.0 μ m	0.37	-0.91**	...
DPC (seed)	9.6 ± 0.4 μ m	0.59	0.49	...
DPC	8.3 ± 0.6 μ m	0.26	0.51	...

^a DPM = *D. phaseolorum* var. *meridionalis*, DPC = *D. phaseolorum* var. *caulivora*, DPS = *D. phaseolorum* var. *sojae*, and PL = *P. longicolla*.

^b Component number and species or varieties separated by it: PC1 = DPM and DPC versus DPS and PL, PC2 = DPM versus DPC, PC3 = DPS versus PL; ** = correlation coefficients significant at $P \leq 0.01$.

D. phaseolorum var. *sojae* isolates DPS99a and CE119 were obtained from infected soybean seed and CE120 was obtained from a soybean plant with stem blight symptoms associated with *Fusarium* spp. (Table 1). These isolates did not produce perithecia under the culture condition used; therefore, the analyses of their morphology were based on the anamorph form only. Colonies showed flosses and ropelike mycelia with white and white-grayish color and yellowish tonalities. The underside of colonies was usually grayish. Black stroma of irregular shape and size frequently were observed scattered in the medium. After 10 days of incubation in 12 h of near-ultraviolet (NUV) light and alternating with 12 h of dark, DPS99a, CE118, and CE119 produced mutic pycnidia exuding α - and, sporadically, β -conidia. Colony development was sustained between 25 and 28°C. The anamorph of *D. phaseolorum* var. *sojae* was different in morphology, color, and stroma of colonies, as well as in the type of conidia produced, from *P. phaseoli*, described as the anamorph of *D. phaseolorum*. These morphological differences previously were reported by Fernández and Hanlin (8) and Yorinori (48). Therefore, we consider the anamorph form of *D. phaseolorum* var. *sojae* to be *P. sojae* Leh. (37) or *P. phaseoli* var. *sojae* according to the criteria followed by Yorinori for the anamorph of *D. phaseolorum* var. *meridionalis* and *caulivora* (49).

Isolates PI5818 and DP4 were defined as *Diaporthe* spp. (Table 1). PI5818 was obtained from stems of senescent soybean plants. This isolate did not fructify when cultured in the conditions detailed above; therefore, it was named a *Diaporthe* sp. based on colony morphology only. Isolate DP4 was obtained from plants showing diffuse stem canker symptoms on the mean portion of the principal stem, and apical senescence. This isolate showed thick strand mycelium and white colonies with white-grayish to green-yellowish tonalities. Stroma with irregular shape and size and pycnidia with α - and, occasionally, β -conidia were observed. Perithecia with prominent peaks were scattered on the stroma in culture or on the stems. Pathogenic symptoms observed in the field could not be reproduced under greenhouse conditions. This isolate was previously classified as a *Diaporthe* sp. based on earlier morphologic and molecular characterization (27) and was not included in the PCA and molecular analysis in this study.

Fig. 2. Grouping of *Diaporthe/Phomopsis* isolates by their morphological characteristics using principal component analysis (PCA). DPM = *D. phaseolorum* var. *meridionalis* (a to g: isolates CE107, CE109, CE110, CE111, CE112, CE113, and CE114, and h: isolate CE108); DPC = *D. phaseolorum* var. *caulivora* (i to j: isolates CE115 and DPC5a, and k: isolate CE116); DPS = *D. phaseolorum* var. *sojae* (l to n: isolates DPS99a, CE119, and CE118); and PL = *P. longicolla* (o to q: isolates CE117, PLClas99b, and PL99.11.1).

P. longicolla isolates CLAS99b, CLAS99a, and CE117 (Table 1) were obtained from stems of soybean plants showing precocious senescence, associated with *Fusarium* spp., *Sclerotium bataticola*, and *Phytophthora sojae*. *Phomopsis longicolla* isolates showed white mycelia, changing to greenish-yellow or a grayish shade with age. Cultures developed black stroma, which usually were large and widespread in the medium. After 25 days in culture, pycnidia with long beaks could be observed spread in the medium and occasionally grouped in clusters. These pycnidia exuded only α -conidia.

PCA. By using the morphologic characteristics described in Table 2, the PCA analysis grouped the isolates of the *Diaporthe/Phomopsis* complex in four distinct taxa: *D. phaseolorum* var. *meridionalis*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *sojae*, and *P. longicolla* (Fig. 2). Three principal components (PC1, PC2, and PC3) explained 85% of the total variability. PC1 discriminated *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora* (SSC-producing isolates) from *D. phaseolorum* var. *sojae* and *P. longicolla* (non-SSC-producing isolates). Characteristics significantly associated with *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora* were compact colonies ($r = 0.72$); white, yellow, to ochre colonies ($r = 0.72$); shrunken to fluffy mycelia ($r = 0.66$); infrequent stroma ($r = 0.72$); perithecia development ($r = 0.91$); solitary perithecia ($r = 0.76$); and clustered perithecia ($r = 0.66$). Meanwhile, characteristics significantly associated with *D. phaseolorum* var. *sojae* and *P. longicolla* were white, gray, or yellow-greenish colony ($r = -0.91$); frequent and long stroma ($r = -0.91$); frequent and scattered stroma ($r = -0.62$); and α - and β -conidia ($r = -0.62$) (Table 2). PC2 discriminated between *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora*. Characteristics specifically and significantly associated with *D. phaseolorum* var. *meridionalis* were tufted or rope-like mycelia ($r = -0.73$), white-tanned colonies ($r = -0.73$), undefined stroma ($r = -0.91$), solitary perithecia ($r = -0.60$), ascus mean length of $36.7 \pm 4.7 \mu\text{m}$ ($r = -0.91$), and ascospore mean length of $10.2 \pm 1.0 \mu\text{m}$ ($r = -0.91$). Characteristics significantly associated with *D. phaseolorum* var. *caulivora* were shrunken to fluffy mycelia ($r = 0.73$) and clustered perithecia ($r = 0.73$) (Table 2). PC3 discriminated between *D. phaseolorum* var. *sojae* (anamorph *P. sojae*) and *P. longicolla* (Table 2). Characteristics significantly associated with *P. sojae* were frequent and scattered stroma ($r = 0.73$), mutic pycnidia ($r = 0.80$), and α - and β -conidia production ($r = 0.73$), whereas pycnidia with long beaks ($r = -0.72$) were significantly associated with *P. longicolla* (Table 2). In the PCA output, the isolates belonging to the same taxon appear closely associated. However, isolates CE108 and CE116 of *D. phaseolorum* var. *meridionalis* and *caulivora*, respectively, had particular morphologic features which distinguished them from the other isolates grouped in the same taxon (Fig. 2).

RAPD analysis. Twenty-five isolates were included in this analysis. DNA from each isolate was amplified using the oligonucleotides indicated above. Amplification patterns resolved in denaturing polyacrylamide gels showed a high number of bands. The total of amplified products was, on average, 75 per primer per isolate, ranging from 250 to 2,000 bp. For comparing isolates, only easily scorable bands were considered and a total of 145 polymorphic bands were analyzed (90 bands for primer OP-AA01 and 55 bands for primer OP-AA06).

Dendrogram obtained with RAPD markers. To estimate the genomic variability of the pathogen isolates, 145 polymorphic RAPD bands were computed in a basic matrix data. Jaccard coefficients were used to establish similarity relationships among fungal genotypes, and a dendrogram showing the distinctive clustering patterns was obtained (Fig. 3). The distortion of the phenogram was assessed by calculation of the correlation coefficient between cophenetic and similarity matrixes. This value (0.997) was found statistically significant by the Mantel test, thus

entirely supporting the phenogram description. Cluster analysis revealed that *D. phaseolorum* var. *meridionalis*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *sojae*, and *P. longicolla* were separated in different groups, with different degrees of relationship within each group (Fig. 3). Although the origins of the four main branches are located at value zero of the Jaccard Coefficient, the similarity within each branch starts at 0.38 for *D. phaseolorum* var. *meridionalis*, 0.48 for *D. phaseolorum* var. *caulivora*, 0.54 for *P. longicolla*, and 0.77 for *D. phaseolorum* var. *sojae*. The control isolates from different taxa were separated and, at the same time, the control belonging to the same taxon was included in the same branch. Therefore, this result, together with the other traits considered in this study, suggests that local isolates included in the same cluster belong to the same taxon. The dendrogram also shows major differences and high similarities among the isolates used in this study. The lower the coefficient of similarity, the higher was the variability within the taxon.

Within *D. phaseolorum* var. *meridionalis* taxon, a subgroup was formed by local isolates CE107, CE109, CE110, CE112, and CE113 with a similarity coefficient of 0.80, and a second subgroup was formed by isolates DPMNa, CE114, and DPMNb with a similarity coefficient of 0.85 (Fig. 3). These later three isolates were controls from Brazil and Paraguay (Table 1). A third subgroup was formed by the local isolate CE111 and the U.S. control DPMS(TC)5, with a similarity coefficient of 0.75. The local isolate CE108 had the lowest coefficient of similarity (0.38) with the rest of the isolates of the group, forming a subgroup of its own (Fig. 3).

Within *D. phaseolorum* var. *caulivora* taxon, local isolates CE116, CE115, and DPC5a grouped with the two U.S. controls, but the level of similitude was 0.87 with U.S. isolate DPCB(IT) and 0.48 with U.S. isolate DPC713(IT) (Fig. 3).

In the *P. longicolla* taxon, local isolates CE117, PLCLAS99a, and PLCLAS99b showed a similarity coefficient of 0.86 among themselves and of 0.54 with the U.S. control PL99.11.1 (Fig. 3).

Within *D. phaseolorum* var. *sojae* taxon, isolates DPS99a, CE119, CE120, and PI5818 were related to control isolate CE118 with a similarity coefficient of 0.77 (Fig. 3). All *D. phaseolorum* var. *sojae* isolates, including the control, were local. Isolate PI5818, which previously could not be classified morphologically, was molecularly included in this taxon.

Pathogenic characterization. The results of pathogenicity trials, expressed as %DP, for the interactions among 14 *Diaporthe* and *Phomopsis* isolates and seven soybean genotypes, carrying four different *Rdc* genes, are shown in Table 3. *D. phaseolorum* var. *sojae* and *P. longicolla* did not produce canker lesions in any of the assayed genotypes, while both *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora* isolates produced stem cankers. The %DP, however, depended on the particular interaction established between each plant genotype and each fungus isolated (Table 3). All isolates of *D. phaseolorum* var. *meridionalis* showed compatible interactions (i.e., produced canker) with cv. RA 702 (66.4 to 91.7% DP), and incompatible interactions with cv. Tracy-M (0 to 3.7% DP) (Table 3). Tracy-M carries two major resistance genes (*Rdc1* and *Rdc2*) and was used as a resistant control, while RA 702 has none of the four *Rdc* genes assayed and was considered a susceptible control. Surprisingly, however, all isolates of *D. phaseolorum* var. *caulivora* showed similar degrees of compatibility with both the susceptible RA 702 (50.8 to 59.8% DP) and the resistant Tracy-M (43.0 to 73.6% DP) cultivars (Table 3). These results mean that *Rdc1* and *Rdc2* together (as in cv. Tracy-M) conferred an almost immune reaction to all assayed isolates of *D. phaseolorum* var. *meridionalis* but were ineffective in conferring resistance to any of the *D. phaseolorum* var. *caulivora* isolates evaluated.

When interacting with genotypes homozygous for single major resistance genes *Rdc1*, *Rdc2*, *Rdc3*, and *Rdc4*, all assayed isolates of *D. phaseolorum* var. *caulivora* showed compatible interactions with all single resistance genes (16.0 to 91.8% DP) (Table 3). On the other hand, when the isolates of *D. phaseolorum* var.

Fig. 3. Dendrogram of *Diaporthe/Phomopsis* isolates based on unweighted pair-group method with arithmetic average obtained using NTSYS program with Jaccard similarity coefficient calculated from random amplified polymorphic DNA. DPM = *D. phaseolorum* var. *meridionalis*, DPC = *D. phaseolorum* var. *caulivora*, DPS = *D. phaseolorum* var. *sojae*, and PL = *P. longicolla*.

meridionalis were inoculated to genotypes homozygous for single major resistant genes, high variability was observed in the number (and grade) of compatible genotype-isolate interactions established. According to the four categories described above, the reactions ranked from incompatible (<15% DP) to highly compatible (≥85% DP). *D. phaseolorum* var. *meridionalis* isolates CE107, CE109, and CE110 were incompatible with genotypes carrying single *Rdc1*, *Rdc2*, *Rdc3*, or *Rdc4* genes (0 to 14.5% DP) (Table 3).

Isolates CE112 and CE113 were compatible with genotypes homozygous for *Rdc1* or *Rdc2* (21.2 to 36.8% DP) but incompatible with genotypes carrying single *Rdc3* or *Rdc4* genes. Isolates CE111 and CE114 were distinct from the previously described group in showing a higher degree of compatibility with *Rdc1* or *Rdc2* (29.4 to 93.8% DP) and a compatible interaction with *Rdc4* in Dowling background (20.2 and 42.0% DP, respectively) (Table 3). These four isolates, compatible with genotypes carrying single *Rdc1* or *Rdc2* genes, were, however, incompatible with genotypes carrying *Rdc1* plus *Rdc2* (such as Tracy-M), proving gene additivity in SSC resistance.

Isolate CE108 differentiated from all others *D. phaseolorum* var. *meridionalis* isolates because it was the only one compatible with *Rdc3* and with *Rdc4* in Hutcheson background (38.0 and 24.9% DP, respectively) (Table 3). This isolate also was compatible with *Rdc4* in Dowling background (44.0% DP) and with *Rdc2* (54.5% DP). Peculiarly, it was incompatible with *Rdc1* (10.5% DP). In addition, when interacting with *Rdc1* and *Rdc2* together (as in Tracy-M), isolate CE108 showed an incompatible interaction (3.5% DP) (Table 3). These responses suggest that *Rdc1* is the main gene responsible for Tracy-M resistance to CE108, although gene additivity could not be totally dismissed for this particular interaction.

The result of the cluster analysis grouping SSC-producing isolates according to their grade of compatibility with soybean genotypes carrying different resistance genes is shown in Figure 4. Two main groups were formed, one with the isolates belonging to *D. phaseolorum* var. *meridionalis* and the other with the isolates belonging to *D. phaseolorum* var. *caulivora*. The similarity coefficient among isolates of the same variety was 56 and 75 for *D. phaseolorum* var. *meridionalis* and *caulivora*, respectively (Fig. 4). The lower the coefficient of similitude, the higher the variability. Thus, for this set of data, the variability observed within *D. phaseolorum* var. *meridionalis* was higher than the

variability within *D. phaseolorum* var. *caulivora*. It should be noted, however, that because *D. phaseolorum* var. *caulivora* was detected only recently in our region (28,31), few local isolates were available for testing. This situation precludes definitive conclusions about its pathogenic variability in our environmental conditions.

Within *D. phaseolorum* var. *meridionalis*, isolates CE107, CE109, and CE110 (incompatible with the four *Rdc* genes assayed) formed a closely related subgroup, with a similarity coefficient of 100, and the same occurred with CE112 and CE113 (compatible with *Rdc1* and *Rdc2* but incompatible with *Rdc3* and *Rdc4*), which formed a second subgroup with the same degree of similarity (Fig. 4). Additionally, isolates of both subgroups were related pathogenically with a similarity coefficient of 89 (Fig. 4). A third subgroup with close similarity was formed by CE111 and CE114 (Brazilian control) with a similarity coefficient of 98. These isolates showed higher levels of compatibility with *Rdc1* and *Rdc2* than isolates of the second subgroup and, in addition, were compatible with *Rdc4* in Dowling background. This later subgroup was 85% similar with CE108 (compatible with *Rdc2*, *Rdc3*, and *Rdc4* but incompatible with *Rdc1*) (Fig. 4). On the other hand, within *D. phaseolorum* var. *caulivora*, local isolates CE115 and CE116 showed similarity coefficients of 95 between them and of 75 with isolate DPCB(IT) (U.S. control).

DISCUSSION

Morphological characterization. Morphologic parameters used to characterize the isolates analyzed in this study were consistent with previous reports on cultural and morphological characteristics of isolates of the *Diaporthe/Phomopsis* complex (8). In the genus *Diaporthe*, stroma morphology and perithecial arrangement could change depending on the substrate and environmental conditions where the fungus is growing. Thus, these characters should be used only under well-defined growth conditions in order to assure reproducibility (8). The results with colony characteristics reported here all were obtained in APGA culture incubated in darkness at $25 \pm 1^\circ\text{C}$. Additionally, fruiting bodies were obtained on cultures or on autoclaved soybean stems on APGA maintained at room temperature and natural day-and-night alternation for 35 to 45 days. Under our experimental conditions, variation in the frequency of stroma formation as well

TABLE 3. Pathogenicity trials for different isolates of the *Diaporthe/Phomopsis* complex, expressed as percentages of dead plants (%DP)

Isolate	Genotype (<i>Rdc</i> genes), %DP ^a						
	Line T-1 (<i>Rdc1</i>)	Line T-2 (<i>Rdc2</i>)	Crockett (<i>Rdc3</i>)	Hutcheson (<i>Rdc4</i>)	Dowling (<i>Rdc4</i>)	Tracy M (<i>Rdc1</i> , <i>Rdc2</i>)	RA 702
<i>D. phaseolorum</i> var. <i>meridionalis</i>							
CE107(2001)	14.5 ± 4.4	8.6 ± 3.8	3.8 ± 1.2	0.0	9.0 ± 3.7	0.0	88.4 ± 2.8
CE109(2001)	10.9 ± 2.7	10.9 ± 2.6	4.1 ± 1.8	0.0	0.0	0.0	91.7 ± 1.9
CE110(2001)	12.5 ± 1.4	10.1 ± 2.7	1.9 ± 1.3	0.0	5.0 ± 3.1	0.0	80.7 ± 4.8
CE112(2001)	25.7 ± 0.5	21.2 ± 1.4	4.1 ± 2.7	0.0	0.0	0.0	81.5 ± 3.3
CE113(2001)	36.8 ± 2.9	35.4 ± 2.4	0.0	0.0	2.0 ± 0.9	0.0	69.8 ± 1.8
CE111(2001)	29.4 ± 6.4	68.9 ± 5.1	4.4 ± 1.9	3.1 ± 1.6	20.2 ± 0.7	3.7 ± 1.8	81.9 ± 2.1
CE114(2001)	74.6 ± 2.3	93.8 ± 2.9	1.0 ± 0.7	0.0	42.0 ± 3.3	0.4 ± 0.4	81.5 ± 2.9
CE108(2001)	10.5 ± 2.9	54.5 ± 1.9	38.0 ± 9.1	24.9 ± 0.8	44.0 ± 1.7	3.5 ± 1.4	66.4 ± 2.8
<i>D. phaseolorum</i> var. <i>caulivora</i>							
CE115(2001)	33.0 ± 1.9	16.0 ± 2.3	ND	28.2 ± 3.2	52.4 ± 1.1	55.2 ± 5.3	52.6 ± 5.6
CE116(2001)	42.4 ± 3.4	31.0 ± 1.1	53.3 ± 1.7	51.2 ± 3.8	70.2 ± 2.9	43.0 ± 9.6	50.8 ± 6.1
DPCB(IT)	51.8 ± 1.8	64.2 ± 2.8	91.8 ± 3.8	53.2 ± 2.1	63.6 ± 2.5	73.6 ± 3.4	59.8 ± 3.4
<i>D. phaseolorum</i> var. <i>sojae</i>							
CE118(2001)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>P. longicolla</i>							
CE117(2001)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PL99.11.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0

^a The %DP (±SE) was obtained 30 days after inoculation as the proportion of plants displaying soybean stem canker symptoms out of the total number of plants assayed. For calculations, a value of 1 was assigned to each dead plant and a value of 0.5 to each infected plant with stem canker symptoms. In all, 54, 36, and 54 seedlings were used in three greenhouse experiments made in 1998 and 1999. Interaction between each plant genotype with each fungus isolate was repeated at least twice in different experiments; ND = not determined.

as in texture and color of colonies among the different varieties of *D. phaseolorum* was observed. Isolates of *D. phaseolorum* var. *meridionalis* usually had undefined stromatic areas. The exception was CE108, which showed small and well defined stroma scattered on the medium. Similarly, infrequent and small stroma were observed for *D. phaseolorum* var. *caulivora* isolates. Conversely, *D. phaseolorum* var. *sojae* and *P. longicolla* formed frequent and well-defined stroma, although of different size and shape. Variation in size and overall morphology of stroma and colony characteristics for *D. phaseolorum* had been previously reported (8,45).

D. phaseolorum var. *meridionalis* production of pycnidia, exuding only α -conidia, was more frequent on soybean stems in APGA than on APGA medium. This result was consistent with previous reports (8,9). Also, as in early reports (1,46), pycnidia were not observed for *D. phaseolorum* var. *caulivora* under our experimental conditions. Other authors reported that α - and β -conidia rarely are produced under natural conditions by *D. phaseolorum* var. *caulivora*, although they sometimes can be produced under laboratory conditions on autoclaved soybean stems in water agar (9). We observed only α - and β -conidia production on *D. phaseolorum* var. *sojae* isolates and on DP4, defined as a *Diaporthe* sp. All *D. phaseolorum* isolates observed in this study showed mutic pycnidia; meanwhile, all isolates of *P. longicolla* showed pycnidia with long beaks. Beaks more than 200 μ m long were cited as characteristic of *P. longicolla* (20).

Under both APGA culture and soybean stems on APGA, *D. phaseolorum* var. *meridionalis* consistently produced solitary perithecia. This perithecial arrangement also was observed on over-wintered soybean stem (data not shown). On the other hand, *D. phaseolorum* var. *caulivora* CE116 showed clustered perithecia on both APGA culture and soybean stems on APGA, but seed-derived isolates DPC5a and CE115 had a less-defined perithecial arrangement, showing both clustered and solitary perithecia. Thus, the perithecia arrangement was more stable for *D. phaseolorum* var. *meridionalis* than for *D. phaseolorum* var. *caulivora*. This result was consistent with the report of Fernandez et al. (9) which

showed that perithecia of *D. phaseolorum* var. *caulivora* form either singly or in caespitose groups; meanwhile, *D. phaseolorum* var. *meridionalis* perithecia are almost always borne singly. For *D. phaseolorum* var. *sojae*, production of perithecia (usually solitary) also was reported on old agar cultures and on over-wintered soybean stems (20). Additionally, variation in perithecia arrangement (solitary or clustered) also was described for this later variety depending on substrate and environmental conditions (8). However, under our experimental conditions, the *D. phaseolorum* var. *sojae* isolates assayed did not develop perithecia. In addition to the substrate and environmental conditions, a genetic component also could be involved in perithecial arrangement and stroma formation in *D. phaseolorum* (8). The tendency to cluster of the perithecia and well-developed stromata were considered to be advanced morphological characteristics in members of the genus *Diaporthe* (45).

Longest and shortest ascus and ascospore length were consistently measured in *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora* isolates, respectively (Table 2). For *D. phaseolorum* var. *caulivora*, the lengths of asci and ascospores measured for the stem isolate CE116 were similar to the values reported in the literature (8,9). However, seed isolates DPC5a and CE115 had longer ascus and ascospore lengths than the stem isolate CE116 (Table 2). Length and width of ascus and ascospore are considered very stable characteristics and were used to differentiate varieties within *D. phaseolorum* (8). Because of the limited number of isolates analyzed in this study, it was not possible to establish if the variability in ascus and ascospore length observed for *D. phaseolorum* var. *caulivora* isolates was significantly associated with the type of infected tissue. Nevertheless, by using morphologic characters, it was possible to discriminate among the three intraspecific taxa within the *D. phaseolorum* species as well as between species within the genus *Phomopsis*.

Molecular characterization. The RAPD analysis produced 145 polymorphic bands. The high number of bands analyzed showed genetic differences among most of the isolates used in this study and the data were sufficient to fingerprint each of the

Fig. 4. Dendrogram of soybean stem canker (SSC) producing isolates of *Diaporthe phaseolorum* based on the category of their interaction with soybean genotypes carrying different major resistance genes to SSC. Grouping was generated with the Cluster procedure, Ward's Method PC-ORD, using Euclidean distance for measuring the similarity among isolates. DPM = *D. phaseolorum* var. *meridionalis*, DPC = *D. phaseolorum* var. *caulivora*.

isolates and to generate a dendrogram based on molecular markers (Fig. 3). In addition to distinguishing interspecific and intra-specific variability of the *Diaporthe/Phomopsis* complex, molecular markers allowed the detection of differences among isolates within the same variety (Fig. 3). Moreover, molecular analysis was able to classify PI5818 as *D. phaseolorum* var. *sojae* (Fig. 3). This latter isolate could not be classified morphologically. Thus, RAPD analysis overcame the limitation of classifying isolates based on morphological characteristics only. Fernández and Hanlin (8), using RAPD markers, were able to differentiate among taxa within the *Diaporthe/Phomopsis* complex, as well as among isolates within *D. phaseolorum* var. *caulivora*. Also, Zhang et al. (51), by using PCR-restriction fragment length polymorphism, detected genomic differences among *D. phaseolorum* var. *caulivora* isolates and *D. phaseolorum* var. *meridionalis* isolates originated from the same areas of Italy and the United States, respectively. In our study, grouping local isolates of the *Diaporthe/Phomopsis* complex in different taxa based on similarity of band patterns corresponded with grouping them based on morphological characters (Figs. 2 and 3). Because of the congruence of both morphological and RAPD groupings and the correspondence among local and control isolates, it was concluded that taxon assignment of the isolates was appropriate. Even when, in our study, a limited number of isolates was used, both the morphologic and molecular analysis allowed the separation of the different taxa of the *Diaporthe/Phomopsis* complex present in the core soybean-producing area of Argentina.

Pathogenic characterization. A remarkable result of the pathogenic trials was that, while the four *Rdc* genes assayed confer different grade of resistance to isolates of *D. phaseolorum* var. *meridionalis*, none of them conferred resistance to any isolate of *D. phaseolorum* var. *caulivora*. Moreover, *Rdc1* and *Rdc2* together, which confer a high level of resistance to *D. phaseolorum* var. *meridionalis*, were ineffective against *D. phaseolorum* var. *caulivora*. Thus, cv. Tracy-M (*Rdc1* and *Rdc2* genes) showed a level of susceptibility to *D. phaseolorum* var. *caulivora* similar to that of cv. RA 702 (without *Rdc* genes) (Table 3). The response observed in Tracy-M was consistent with previous reports which rated it as resistant to pathogenic isolates from Mississippi and Tennessee (southern isolates) but susceptible to isolates from Iowa, Indiana, and Ohio (northern isolates) (11,14,15). A similar response also was reported for cvs. Blackhawk and Hawkeye, classified as very susceptible to northern isolates but resistant to southern isolates of *D. phaseolorum* (3,19).

Morphological, biological, and ecological differences, as well as physiological capacity to induce stem canker, made it possible

to distinguish between northern and southern isolates in the United States (3,11,13,14,16). Based on these differences, the causal agent of northern and southern stem canker were named *caulivora* and *meridionalis*, respectively; first as forma specialis (19,22,23) and then as varieties (8,36) at the subspecific level of *D. phaseolorum*. In addition, the two major dominant alleles for SSC resistance, *Rdc1* and *Rdc2*, found in cv. Tracy-M, and their separation in lines homozygous for each single dominant allele, was accomplished by using a *D. phaseolorum* isolate from Mississippi (17,18), presumably belonging to *D. phaseolorum* var. *meridionalis*. Later, the identification of *Rdc3* and *Rdc4* in cvs. Crockett and Dowling, respectively, was performed using a southern isolate from Texas (5). Then, an isolate identified as *D. phaseolorum* var. *meridionalis* was used to determine that *Rdc4* was also the source of resistance to SSC in cv. Hutcheson (43). The identification of soybean resistance genes was done with southern U.S. isolates of *D. phaseolorum*, later named var. *meridionalis*; therefore, it should not be unexpected that these genes do not confer resistance to isolates of *D. phaseolorum* var. *caulivora*. Moreover, according to the virulence and avirulence gene concept (42), our results, together with results reported in the literature (3,11,14–16,19,44), strongly suggest that genes for resistance to *D. phaseolorum* var. *meridionalis* are different from genes for resistance to *D. phaseolorum* var. *caulivora*. Practically, it means that selecting germ plasm for resistance to *D. phaseolorum* var. *meridionalis* will not guarantee resistance to *D. phaseolorum* var. *caulivora*. Thus, to avoid future misleading in breeding for SSC resistance, we propose to rename the known four major genes conferring resistance to *D. phaseolorum* var. *meridionalis*, *Rdc1*, *Rdc2*, *Rdc3*, and *Rdc4*, as *Rdm1*, *Rdm2*, *Rdm3*, and *Rdm4*, respectively.

It was suggested that there is no overlap in the geographical range of the two varieties of *D. phaseolorum* causing SSC in the United States (9). On the contrary, we documented the coexistence of both varieties of *D. phaseolorum* (i.e., *meridionalis* and *caulivora*) in the core soybean-producing area of Argentina (28,31). Ecological differences, mainly in temperatures, between northern and southern soybean-producing areas of the United States could explain the geographical separation of *D. phaseolorum* var. *meridionalis* and *caulivora* (16). Conversely, the environmental conditions prevalent in the core soybean-producing area of Argentina could favor their coexistence. However, the influence of the environmental factors on the prevalence of one or the other variety of *D. phaseolorum*, as well as on the possibility of interbreeding between them, is an area that deserves additional research.

TABLE 4. Plant–fungus interaction among soybean genotypes carrying different resistance genes for stem canker and *Diaporthe phaseolorum* isolates

Isolates	Genotype (<i>Rdc</i> genes) ^a						RA 702
	Line T-1 (<i>Rdc1</i>)	Line T-2 (<i>Rdc2</i>)	Crockett (<i>Rdc3</i>)	Hutcheson (<i>Rdc4</i>)	Dowling (<i>Rdc4</i>)	Tracy M (<i>Rdc1</i> , <i>Rdc2</i>)	
<i>D. phaseolorum</i> var. <i>meridionalis</i>							
CE107(2001)	–	–	–	–	–	–	+++
CE109(2001)	–	–	–	–	–	–	+++
CE110(2001)	–	–	–	–	–	–	++
CE112(2001)	+	+	–	–	–	–	++
CE113(2001)	+	+	–	–	–	–	++
CE111(2001)	+	++	–	–	+	–	++
CE114(2001)	++	+++	–	–	+	–	++
CE108(2001)	–	++	+	+	+	–	++
<i>D. phaseolorum</i> var. <i>caulivora</i>							
CE115(2001)	+	+	ND	+	++	++	++
CE116(2001)	+	+	++	++	++	+	++
DPCB(IT)	++	++	+++	++	++	++	++

^a Plant–fungus interactions: – = incompatible, 0 to 14.9% dead plants (DP); + = compatible, 15 to 49.9% DP; ++ = very compatible, 50 to 84.9% DP; +++ = highly compatible, 85 to 100% DP. Symbols enclosed by squares indicate the existence of cross reaction between isolate CE108 versus isolates CE112, CE113, CE111, or CE114, when interacting with soybean genotypes homozygous for *Rdc1* versus genotypes homozygous for *Rdc3* and *Rdc4*; ND = not determined.

Another striking result obtained in this study was the variability in the virulence of different isolates of *D. phaseolorum* var. *meridionalis* when interacting with soybean genotypes carrying different SSC resistance genes. The observed variability in the plant–fungus interaction is summarized in Table 4, according to the four categories established in the rank of compatibility stated above. With the differential soybean genotypes (and *Rdc* genes) used, four subgroups of *D. phaseolorum* var. *meridionalis* isolates of distinct virulence could be clearly identified. One subgroup was formed by isolates CE107, CE109, and CE110, which were avirulent to all *Rdc* genes assayed. These isolates were, however, virulent to the susceptible cv. RA 702 (without *Rdc* genes) (Table 4). In the pathogenicity dendrogram, this group was related with a similarity coefficient of 100 (Fig. 4). The second subgroup, formed by isolates CE112 and CE113, was virulent to *Rdc1* and *Rdc2* genes but avirulent to *Rdc3* and *Rdc4* genes (Table 4). This second subgroup also was related 100% in the cluster analysis (Fig. 4). The third subgroup, formed by isolates CE111 and CE114, differentiated from the second by being virulent to the *Rdc4* gene in Dowling background. Finally, isolate CE108 was unique in being virulent to genes *Rdc2*, *Rdc3*, and *Rdc4* (Hutcheson and Dowling backgrounds) but avirulent to *Rdc1*. CE108 was the only isolate morphologically and molecularly grouped as *D. phaseolorum* var. *meridionalis*, compatible with genes *Rdc3* and *Rdc4*. Thus, the high level of resistance to *D. phaseolorum* var. *meridionalis* conferred individually by genes *Rdc3* or *Rdc4* clearly was overcome by isolate CE108. Additionally, isolate CE108 crossreacted with isolates CE112, CE113, CE111, and CE114 when interacting with soybean genotypes homozygous for *Rdc1* versus genotypes homozygous for *Rdc3* and *Rdc4* (Table 4). The existence of cross reaction is a clear indication of specificity between host and pathogen. Interestingly, the local isolate CE108 also was differentiated from the others within the *D. phaseolorum* var. *meridionalis* taxon, in both the PCA and the molecular analysis (Figs. 2 and 3, respectively).

The results summarized in Table 4 show that, with the set of plant differentials used, it was possible to distinguish at least four different combinations of virulence and avirulence genes in the set of *D. phaseolorum* var. *meridionalis* isolates assayed. This demonstrated that at least four different local physiological races of *D. phaseolorum* var. *meridionalis* are present in the core soybean-producing area of Argentina. As far as we know, the existence of physiological races of *D. phaseolorum* var. *meridionalis* has not been reported previously for South America. Selective pressure due to deployment of resistant host cultivars may have changed the frequency of the virulence or avirulence genes within the population of *D. phaseolorum* var. *meridionalis* under field conditions. Thus, new races virulent to a particular resistance gene could be selected on cultivars carrying that gene. The more widely resistant cultivars (and *Rdc* genes) were grown, the greater the selection pressure, and the shorter the time for the pathogen to develop variants (7). Also, a new variant of the pathogen could be virulent to one or more previously resistant genes but avirulent to another. This seems to be the case with CE108. Even though there is no public record, it is known that cv. Hutcheson has been used extensively in local breeding programs as a source of resistance to SSC. So it is very possible that *Rdc4* is widely incorporated in commercial soybean cultivars grown in our region, favoring the selection of new races of the pathogen virulent to it.

It is noteworthy that clustering isolates based on their pathogenicity showed high correspondence with clustering them based on their genomic variability (Figs. 3 and 4). Both pathogenicity trials and molecular analysis grouped SSC producing varieties of *D. phaseolorum* (i.e., *caulivora* and *meridionalis*) in different taxa. Also, within the *D. phaseolorum* var. *meridionalis* taxon, isolates CE107, CE109, CE110, CE112, and CE113 were closely related by both pathogenic and molecular clustering, even though CE112 was considered molecularly identical to CE107, CE109,

and CE110 (Fig. 3) but was pathogenically grouped with CE113 (Fig. 4). Isolates CE111 and CE114 were closely related pathogenically (Fig. 4) but, molecularly, these isolates clustered in different subgroups (Fig. 3). CE108 was separated from all of the others belonging to *D. phaseolorum* var. *meridionalis* in both pathogenic and molecular analyses, and this separation was particularly evident in the molecular analysis (Fig. 3). On the other hand, the pathogenic and molecular grouping of *D. phaseolorum* var. *caulivora* isolates CE115, CE116, and DPCB(IT) were very similar (Figs. 3 and 4). A perfect match between both dendrograms should not be expected. First, the identity and quantity of isolates of *D. phaseolorum* var. *meridionalis* and *caulivora* included in each analysis were not the same. Second, *P. longicolla* and *D. phaseolorum* var. *sojae* isolates were not used in the pathogenic analysis because they did not produce SSC. Third, although isolates were similar pathogenically, their genomes may be quite distinct, requiring only identity in genes involved in the virulence and avirulence response toward differential plant genotypes. For the same reason, new virulence or avirulence factors can be discerned when a given race is tested on a larger set of differentials (7). In our experiment, the inclusion of a second cultivar carrying the *Rdc4* gene (i.e., Dowling) allowed the identification of another virulence gene (and physiological race) present in CE111 and CE114, which were virulent to *Rdc4* only in the Dowling background (Tables 3 and 4). Additionally, the fact that some isolates of *D. phaseolorum* var. *meridionalis* showed a different degree of compatibility when interacting with the same resistance gene in different backgrounds (e.g., *Rdc4* gene in Dowling or in Hutcheson) suggests the existence of another gene or genes that are associated with the assayed ones and contribute to the resistance to SSC in soybean.

On the whole, the results reported here show that, in the environmental conditions prevalent in the core soybean-producing area of Argentina, the pathogenic variability of *D. phaseolorum* is higher than previously recognized. Hence, testing a single plant–fungus interaction would not cover the pathogenic variability observed between *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *caulivora* or within *D. phaseolorum* var. *meridionalis*. Future work aimed at the identification of molecular markers linked to the host resistance and fungus virulence genes, and their eventual cloning, will help to better understand the gene-for-gene system in this plant–fungus interaction, and to redesign strategies for durable incorporation of SSC resistance.

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