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Genetic diversity analysis of isolates of the fungal bean pathogen *Pseudocercospora griseola* from central and southern Brazil

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ABSTRACT. Planting resistant varieties is the most effective control measure against the angular leaf spot of dry beans, a fungal disease caused by *Pseudocercospora griseola*. However, dry bean varieties with durable resistance are not easily obtained. Knowledge about the genetic variability of the pathogen population is key for the success of dry bean breeding programs aimed at developing resistant materials, but finding suitable operationally simple and genetically accurate markers is not an easy task. The aim of this study was to assess the suitability of the ISSR-PCR technique to quantify the genetic variability of *P. griseola* isolates. Total

DNA of 27 *P. griseola* isolates from Goiás, Minas Gerais, Espírito Santo, and Paraná States was extracted and amplified using specific primers for ISSR. Using cluster analysis, 27 genotypes were identified. The ISSR-PCR technique was suitable for assessing intraspecific variability of *P. griseola*. The ISSR-PCR marker was found to be highly sensitive to genetic variation and can aid in elucidating the genetic structure of the population of this plant pathogen as a support tool for the dry bean breeding programs.

Key words: Genetic diversity; Angular leaf spot; Beans; *Phaseolus vulgaris*

INTRODUCTION

Angular leaf spot is a destructive disease of dry beans (*Phaseolus vulgaris* L.) caused by the fungus *Pseudocercospora griseola* (Sacc.) Crous and U. Braun (Crous et al., 2006). Angular leaf spot is primarily a disease of the aerial part of the plant and occurs throughout the world where this legume is grown, often causing serious crop losses. It is an extremely important problem in Brazil (Stenglein et al., 2003; Vieira et al., 2006), the largest producer of common beans in the world (FAO, 2008). Disease control through farming practices is virtually impossible, and the high variability of the pathogen makes it difficult to identify bean cultivars with broad and long-lasting resistance. Understanding this variability is crucial to the development of strategies aimed at obtaining resistant varieties (Sartorato et al., 1996; Faleiro et al., 2001; Sartorato, 2004).

P. griseola has no known sexual cycle. Direct observations of the occurrence of different reproduction modes are difficult for many pathogens because of their small size and variable life forms (Samils et al., 2001). Most fungi can reproduce asexually by the mitotic production of spores; however, a large portion can also reproduce sexually. In fact, recent results from genomic and population investigations conducted in the last decade indicate that all fungal populations seem to undergo some form of recombination. Furthermore, all fungal genomes have some genes that are involved in sex (e.g., mating type genes) (Taylor and Berbee, 2006). The identification of mating and meiotic genes does not provide conclusive proof of actual sexual recombination because the functions of these genes may include additional pleiotropic roles that prevent their loss by genetic drift. However, a population genetic approach with the analysis of the pattern of genetic variability using high-resolution molecular markers allows inferences about the occurrence of recombination in the life history of the pathogen (Rosenblum et al., 2010).

Few molecular techniques have been used to study genetic variation in *P. griseola*. The genetic diversity of *P. griseola* was initially evaluated by Alvarez-Ayala and Schwartz (1979) by injecting spores into resistant and susceptible bean cultivars. Pastor-Corrales and Jara (1995) estimated the genetic diversity of *P. griseola* and its co-evolution with the common bean in Latin America by developing differential cultivars and using RAPD (random amplification polymorphic DNA) molecular markers. Studies related to the classification of isolates in races using differential varieties and characterization by RAPD have shown that *P. griseola* is extremely diverse (Mahuku et al., 2002; Sartorato, 2004). Stenglein and Balatti (2006) estimated the genetic diversity of 45 *P. griseola* isolates using differential varieties as well as RAPD and ISSR (inter-simple sequence repeat) molecular techniques. The combination of RAPD-ISSR polymorphism defined 18 haplotypes.

The ISSR technique was originally developed by Zietkiewics et al. (1994). This technique involves a sequence of microsatellites as primers in PCR to generate multi-allelic markers. It combines the advantages of microsatellites and AFLP (amplified fragment length polymorphism) markers over the universality of RAPD. They are highly polymorphic and useful in the study of genetic diversity, phylogeny, molecular characterization, and evolutionary biology (Readdy et al., 2002). This tool was successfully used in studies of fungal populations, insects and vertebrates (Wolfe, 2005). Studies by Tymon and Pell (2005) allowed the estimation of the genetic diversity of 30 isolates of the entomopathogenic fungus *P. neoaphidis* of different geographic origins to be calculated using ISSR, ERIC and RAPD with a larger number of polymorphic bands obtained from ISSR.

The aim of this study was to determine if ISSR-PCR is suitable for the detection of genetic polymorphisms, which would enable the estimation of the amount of intraspecific genetic variability of *P. griseola* isolates.

MATERIAL AND METHODS

Microorganisms and mycelium culture

Twenty-seven isolates of *P. griseola* (Table 1) were kindly donated by Dr. Everaldo Gonçalves de Barros (Department of General Biology, Universidade Federal de Viçosa, Viçosa, MG, Brazil) and were from the fungus collection of EMBRAPA, National Center of Rice and Bean Research, Department of Phytopathology and Molecular Biology. Maintenance of the isolates and production of mycelium were performed as described by Lima et al. (2010).

Table 1. *Pseudocercospora griseola* isolates and origin.

Isolate	Origin
29-3	Lambari, MG
97-2	Coimbra, MG
158-1	Afonso Cláudio, ES
592-3	Anápolis, GO
397	Santo Antônio de Goiás, GO
410	Santo Antônio de Goiás, GO
419-3	Lapa, PR
419-6	Lapa, PR
478-2	Pato Branco, PR
811	Damolândia, GO
836	Damolândia, GO
838	Damolândia, GO
848	Damolândia, GO
858	Damolândia, GO
868	Damolândia, GO
1018	Ponta Grossa, PR
1033	Santo Antônio de Goiás, GO
1038	Santo Antônio de Goiás, GO
1190	Santo Antônio de Goiás, GO
1191	Santo Antônio de Goiás, GO
1205	Goiânia, GO
1206	Goiânia, GO
1207	Goiânia, GO
1209	Goiânia, GO
1213	Goiânia, GO
1218	Unaí, MG
1285	Santo Antônio de Goiás, GO

MG = Minas Gerais; GO = Goiás; ES = Espírito Santo; PR = Paraná.

Total DNA extraction

The extraction of total nucleic acids was performed as described by Lima et al. (2010). To digest total RNA, 3 µL RNase A (10 µg/mL) was added and the solution was incubated for 30 min at 37°C.

ISSR sequences: amplification, electrophoresis and data analysis

The ISSR region was amplified by PCR according to Stenglein and Balatti (2006), with modifications. Previously, 100 primers were tested and eight of these primers were chosen (Table 2). PCRs were performed in a volume of 25 µL containing 50 ng total DNA, 200 µM dNTPs, 0.5 U Taq DNA polymerase, 50 pmol primers and 2.5 µL 10X buffer. The amplifications were performed in a PTC-100 thermocycler (MJ Research Inc.), programmed to perform an initial denaturation of 94°C for 3 min and 40 cycles at 92°C for 1 min, 2 min at the primer annealing temperature, which varied according to the primer used (Table 2), and at 72°C for 2 min, followed by a final extension at 72°C for 7 min.

Table 2. Primers used for amplification of ISSR sequences.

Primers	Sequence (5'-3')	Annealing temperature (°C)	DNA bands	
			Total	Polymorphic
UBC 809	AGAGAGAGAGAGAGAGG	53	5	4
UBC 836	AGAGAGAGAGAGAGAGYA	50	8	6
UBC 842	GAGAGAGAGAGAGAGAYG	48	8	2
UBC 880	GGAGAGGAGAGGAGA	53	7	7
UBC 888	BDBCACACACACACACA	50	12	9
UBC 889	DBDACACACACACACCA	52	7	5
UBC 890	VHVGTGTGTGTGTGT	57	4	4
UBC 891	HVHTGTGTGTGTGTG	55	5	3

Y = (C,T); B = (C,G,T); D = (A,G,T); H = (A,C,T); V = (A,C,G).

To ensure reproducibility of the DNA fragments amplified, all PCRs were performed in duplicate for each isolate, and reactions without DNA were performed to determine if contaminant DNA was present.

The amplification products were analyzed by electrophoresis on a 1.5% agarose gel in 1X TBE buffer under a constant voltage of 4 V/cm. These fragments were visualized by ethidium bromide (0.5 µg/mL) staining and UV light exposure and digitized using the Eagle Eye II photo documentation system. To estimate the size of the amplified DNA fragments, a 1-kb DNA ladder was used as molecular size marker.

For data analysis, each band with a different electrophoretic mobility was assigned a position number and a mark of 1 or 0 based on the presence or absence of the band. Only reproducible bands were considered for analysis. Bands common to all isolates were incorporated into the analysis. The genetic distance between isolates was calculated using Nei's genetic distance (Nei, 1973). Based on this coefficient, a dendrogram was constructed by the unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering algorithm using the POPGENE program (Yeh et al., 1999). The average Nei's gene diversity (H_S) across all loci was estimated (Nei, 1987). The genotypic diversity was estimated by the Shannon-Wiener H' index (Hill, 1973).

RESULTS

Of the 100 primers screened, eight ISSR primers that produced clear and reproducible bands were selected for amplifying 27 *P. griseola* DNA samples. At least four bands were detected for each primer with all isolates. The maximum number of bands was 12, depending on the genotype and primers used (Table 2).

The ISSR-PCR analysis of the total DNA of *P. griseola* isolates using ISSR primers (Table 2) resulted in a total of 56 loci, with sizes ranging approximately between 300-2000 bp. Overall, 40 (71.4%) of these bands were polymorphic, and bands with low resolution were not used in the analysis. The ISSR technique revealed a large number of genotypes, 27. The profiles of bands of some isolates obtained with the UBC 836 and UBC 888 primers are shown in Figure 1. The oligonucleotide sequences of primers and the multiple band pattern observed are summarized in Table 2.

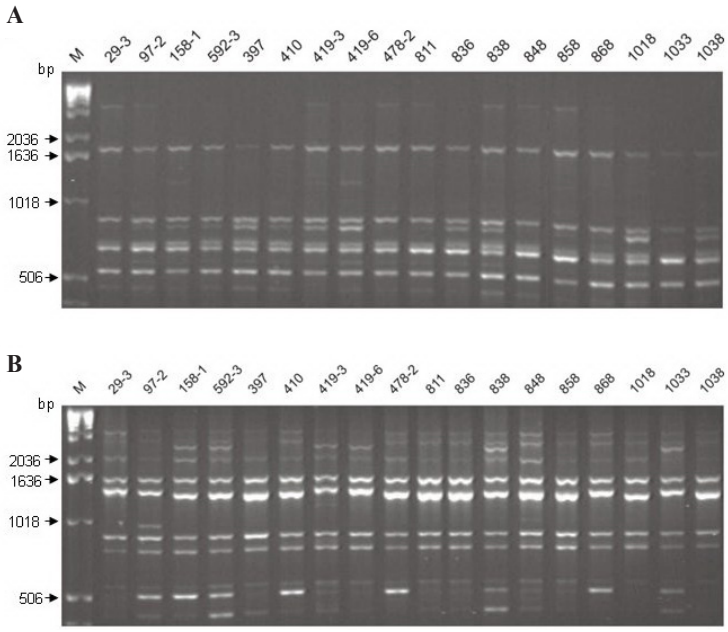

Figure 1. Fingerprints of some *Pseudocercospora griseola* isolates: **A.** using the UBC 836 and **B.** using the UBC 888 ISSR primers. *Lane M* = 1-kb DNA ladder as a molecular size marker. Numbers indicate the isolates in Table 1.

Cluster analysis of ISSR data resulted in a dendrogram that separated the isolates into 27 genotypes (Figure 2). However, it was not possible to determine a clear cluster in the dendrogram by the UPGMA method. These results also showed the inability to identify a structure of isolates according to the place of origin.

Genetic diversity was measured as the Shannon-Wiener H' index and the average H_S index for the 56 loci of ISSR. H_S was estimated to be 0.21 ± 0.18 (± standard deviation) and Shannon's information index was maximum, 3.3. Gene and genotypic diversity were high, indicating that each isolate represents a haplotype.

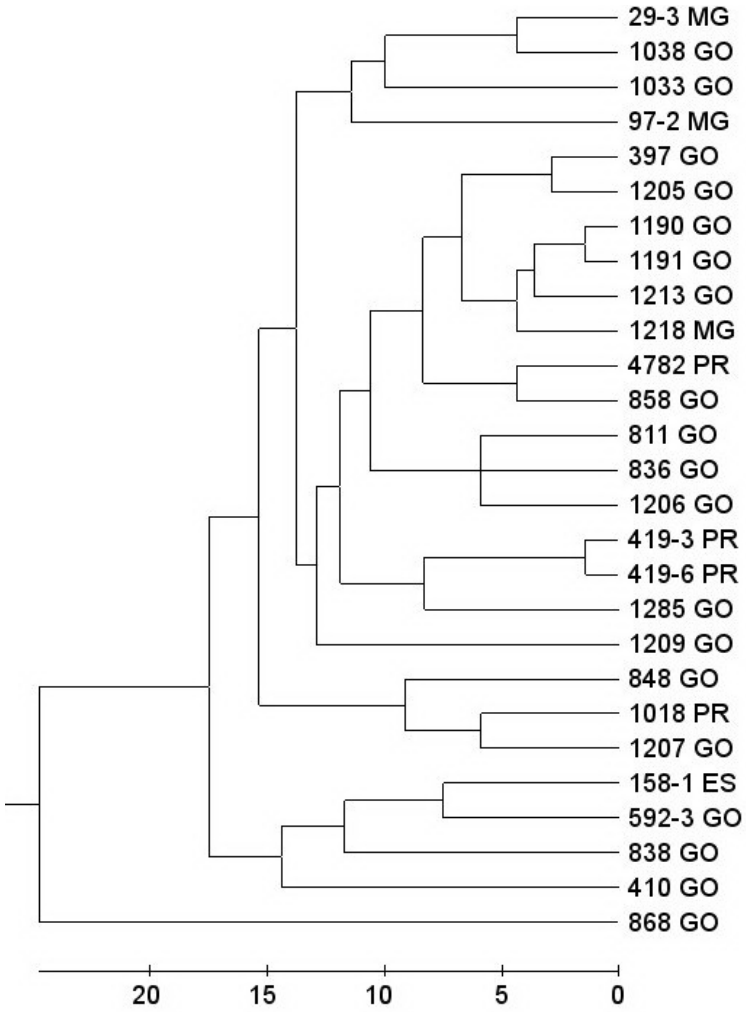

Figure 2. Dendrogram obtained using the UPGMA method showing the genetic relationships among *Pseudocercospora griseola* isolates based on ISSR-PCR typing. Numbers indicate the isolates in Table 1. MG = Minas Gerais; GO = Goiás; ES = Espírito Santo; PR = Paraná.

DISCUSSION

The high level of polymorphic haplotypes detected demonstrated that the ISSR-PCR technique was suitable for the discrimination of *P. griseola* genotypes. The advent of PCR technology led to important achievements in genome analysis. Several molecular methods have been used to analyze the diversity of plant pathogens at the genome level, such as RAPD (Chadha and Gopalakrishina, 2005; Motlagh and Anvari, 2010), AFLP (Zhong and Steffenson, 2001; Douhan et al., 2009) and ISSR (Stenglein and Balatti, 2006; Bayraktar and Dolar, 2010). However, ISSR has important advantages when compared to other methods. The ISSR

technique can generate more polymorphic amplicons than the AFLP technique (Meng and Chen, 2001). Furthermore, the ISSR technique requires a smaller amount of genomic DNA than does the AFLP technique, and does not require the process of enzymatic digestion and ligation. Also, the procedure of the ISSR technique has a number of advantages over other molecular techniques such as RAPD. The ISSR technique is more specific than the RAPD technique due to the use of longer oligonucleotide sequences, allowing more stringent annealing conditions in PCR amplification. It was reported that this technique is more reproducible than RAPD and has higher levels of polymorphism (Reddy et al., 2002).

This study has shown that isolates of *P. griseola* have a high genetic variability when assessed by ISSR markers. The origin of such wide variability in *P. griseola* is unclear because this fungus has no known sexual cycle. However, mechanisms such as mutation, recombination, parasexual reproduction, and migration may contribute to their great diversity (Mahuku et al., 2002). The ISSR-PCR marker was highly sensitive to genetic variation and can aid in elucidating the genetic structure of the population of this plant pathogen as a support tool for dry bean breeding programs. Thus, ISSR markers are a good choice for DNA fingerprinting in *P. griseola*.

Studies by Sartorato (2004), with the objective of using RAPD to estimate the genetic diversity of 96 *P. griseola* isolates obtained at two collection sites in the State of Goiás, showed high genetic diversity among isolates and were also not able to group them according to their geographic origin. The estimated genetic diversity among *P. griseola* isolates was also reported in other studies of this fungus. Stenglein and Balatti (2006) estimated the genetic diversity of 45 *P. griseola* isolates from Argentina using a variety of differential bean cultivars and ISSR- and RAPD-type markers. The combination of RAPD and ISSR defined 18 genotypes; however, the test of the differential series identified only 13 genotypes. Here, we identified 27 genotypes by the ISSR technique.

In summary, ISSR was able to reveal the high genetic variability of *P. griseola* isolates, revealed by the capacity to detect isolate polymorphisms. The observed genetic diversity can aid in the development of appropriate strategies to obtain bean varieties resistant to angular leaf spot.

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