RAPD AND SCAR MARKERS LINKED TO RESISTANCE GENE TO ANGULAR LEAF SPOT IN COMMON BEANS

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(Accepted for publication on 21/09/2000)

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SARTORATO, A., NIETSCHE, S., BARROS, E.G. & MOREIRA, M.A. RAPD and SCAR markers linked to resistance gene to angular leaf spot in common beans. Fitopatologia Brasileira 25:637-642. 2000.

ABSTRACT

Angular leaf spot, caused by the fungus *Phaeoisariopsis griseola*, is one of the most important bean (*Phaseolus vulgaris*) diseases in Brazil, leading to losses as high as 70%. The pathogen has been demonstrated to be highly variable. As a result, any breeding program is dependent on the study of the pathogen variability and on the identification of new resistant genes that might be transferred into commercially well-adapted cultivars. The objectives of this study were to determine the inheritance of disease resistance and to identify RAPD and SCAR markers linked to angular leaf spot resistant gene in the cross between the Middle American cultivars Mexico 54 (resistant) and Ruda (susceptible). The parents, F_1, F_2 and backcross-derived populations were inoculated with the pathotype 63-19 of *P. griseola* under controlled greenhouse conditions. Results indicate that a single dominant gene controls resistance in Mexico 54 showing a segregation ratio of 3:1 in the F_2, 1:0 in the backcross to Mexico 54 and 1:1 in the backcross to Ruda. Three RAPD markers were identified in the F_2 population, $OPN02_{890}$, $OPAC14_{2400}$ and $OPE04_{650}$. These markers were mapped in coupling phase at 5.9, 6.6 and 11.8 cM from the resistant gene, respectively. The $OPN02_{890}$ fragment was transformed into a SCAR marker. The polymorphism observed after amplification was identical to the one revealed with the corresponding RAPD marker.

Key words: *Phaseolus vulgaris*, *Phaeoisariopsis griseola*, molecular marker.

RESUMO

Marcadores RAPD e SCAR ligados a genes de resistência à mancha angular no feijoeiro comum

A mancha angular do feijoeiro comum (*Phaseolus vulgaris*), cujo agente causal é o fungo *Phaeoisariopsis griseola*, é uma das principais doenças desta cultura no Brasil, causando perdas de até 70% na produção de grãos. O patógeno tem demonstrado ser altamente variável. Assim, todo programa de melhoramento depende do estudo desta variabilidade e da identificação de novos genes de resistência que precisam ser transferidos para cultivares comerciais. O objetivo deste estudo foi o de determinar a herança da resistência e identificar marcadores RAPD e SCAR ligados a gene de resistência à mancha angular no cruzamento entre as cultivares Mesoamericanas México 54 (resistente) e Rudá (suscetível). Os genitores, F_1, F_2 e as plantas derivadas de retrocruzamento foram inoculadas com o patótipo 63-19 de *P. griseola* em condições de casa de vegetação. Os resultados indicaram que a resistência da cultivar México 54 é controlada por um único gene dominante. Foi observada uma segregação de 3:1 na população F_2, 1:0 nas plantas do retrocruzamento para a cultivar México 54 e de 1:1 nas plantas do retrocruzamento para a cultivar Rudá. Três marcadores RAPD foram identificados na população F_2 $OPN02_{890}$, $OPAC14_{2400}$ e $OPE04_{650}$. Estes marcadores foram mapeados em acoplamento a uma distância de 5,9, 6,6 e 11,8 cM do gene de resistência, respectivamente. O fragmento $OPN02_{890}$ foi transformado em um marcador do tipo SCAR. O polimorfismo observado após a amplificação com este marcador foi idêntico àquele revelado com o correspondente marcador RAPD.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is one of the most important cultivated leguminous crops in Brazil. It is the host of a large number of diseases including those caused by fungus, bacteria, viruses and nematodes. Angular leaf spot (ALS), caused by the fungus *Phaeoisariopsis griseola* (Sacc.) Ferr., is widespread in beans grown throughout Brazil. Losses due to the disease can be as high as 70% (Mora-Brenes *et al.*, 1983; Sartorato & Rava, 1992). Control methods of the disease include cultural practices, chemical control and genetic resistance. The development and use of resistant cultivars

*CNPq fellowship

currently offers the only economical means for disease control, especially for medium and small farmers. However, the extensive pathogenic variability of *P. griseola* (Sartorato *et al.*, 1991; Pastor Corrales & Jara, 1995; Nietsche, 1997) prevents the development of such resistant cultivars. As a consequence, the lifetime of a resistant cultivar is very short, not more than a few years in favorable conditions for disease development. This is the main reason why breeding programs frequently need to develop cultivars with new sources of resistance.

Determination of resistance gene number and their inheritance mode in *P. vulgaris* to *P. griseola* is very important for developing new resistant cultivars. Resistance to this pathogen has been attributed to one, two or three dominant or recessive genes (Barros *et al.*, 1957; Cardona-Alvarez, 1962; Santos Filho *et al.*, 1976; Singh & Saini, 1980; Sartorato *et al.*, 1993; Carvalho *et al.*, 1997; Ferreira, 1998).

Molecular markers in a plant-breeding program have been used to monitor the transfer of specific genes from one cultivar to another. The Random Amplified Polymorphic DNA (RAPD) markers have been used to identify and select genotypes with resistance genes (Haley *et al.*, 1993; Haley *et al.*, 1994; Adam-Blondon *et al.*, 1994; Young & Kelly, 1994; Johnson *et al.*, 1995; Alzate-Marin, 1996; Carvalho *et al.*, 1997; Ferreira, 1998). Such genes can be used to develop bean cultivars resistant to diseases. However, RAPD has been known for its sensitivity to many factors that limit reproducibility mainly among different laboratories (Penner *et al.*, 1993). The use of Sequence Characterized Amplified Region (SCAR) produces genetic markers that are highly specific, which may overcome this problem.

The objective of this study was to determine the inheritance of resistance to bean angular leaf spot in Mexico 54 and to identify linked RAPD and SCAR molecular markers.

MATERIAL AND METHODS

The experiments were performed in the greenhouse and in the Plant Molecular Biology laboratories of the Biotechnology Institute (BIOAGRO) of the Federal University of Viçosa, MG, Brazil. In all experiments pathotype 63-19 (Nietsche, 1997) of *P. griseola* was used.

Disease inheritance

For the inheritance studies, Middle American cultivars Mexico 54 (resistant) and Ruda (susceptible) were crossed. Seeds from the parents (30 plants), F_1 (20 plants) F_2 (167 plants), and backcross (BC) (46 plants for BC_S and 29 plants for BC_R) populations were treated with Rhodiauram (Thiran), pre-germinated and sown, three seeds/pot, in 3 kg pot containing non sterilized soil. Eighteen days after planting, all plants were inoculated with a spore suspension of 2 x 10^4 conidia.ml^{-1}. Inoculated plants were incubated for 48 h in a humid chamber (22 ± 1° C and 95% relative humidity). After this period, plants were transferred to greenhouse benches (28 ± 2° C). Disease severity was evaluated 12 days after inoculation using a 1-to-9 disease scale (CIAT, 1987). Plants that exhibited up to 15% of degree 4 were considered resistant; other plants were susceptible.

RAPD markers

The Bulked Segregant Analysis (BSA) technique (Michelmore *et al.*, 1991) was used to construct DNA bulks from resistant and susceptible F_2 plants. Each bulk contained DNA from eight resistant or susceptible plants. The DNA was extracted from bean leaves according to Doyle & Doyle (1987). Samples were amplified by the RAPD technique (Williams *et al.*, 1990), using decamer primers from Operon Technologies (Alameda, CA, USA) and thermocycler model 9600 from Perkin-Elmer (Norwalk, CT, USA). Each amplification reaction of 25 µl contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.8 mM $MgCl_2$, 0.1 mM of each dNTP, 0.4 µM of primer, one unit of *Taq* DNA polymerase and 25 ng of DNA. Each amplification cycle consisted of the following steps: 15 s at 94 °C, 30 s at 35 °C and 60 s at 72 °C. After 40 cycles, samples were submitted to 7 min at 72 °C and, finally kept at 4 °C. Amplification products were separated on 1.2% agarose gels containing 0,2 µg of ethidium bromide /ml of gel, immersed in TBE (90 mM Tris-borate buffer, 1 mM EDTA, pH 8.0). DNA bands were visualized under UV light and photographed with the Eagle Eye II system (Stratagene, La Jolla, CA, USA).

SCAR marker

The RAPD band $N02_{890}$ was excised from the agarose gel, purified with the Glass Max™ DNA Isolation Matrix System (BRL) and cloned in the vector pGEM-T Easy (Promega). White colonies were grown in 2 ml LB medium containing 100 µg/ml ampicilin and the plasmid was purified with the QIA Prep Spin Miniprep kit (Qiagen). Both ends of the clone were partially sequenced by automated sequencing ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) using the M13 universal primers. The sequence data was used to design two primers each containing 20 nucleotides including the sequence of the original RAPD primer. The primers were synthesized by BioSynthesis.

PCR reaction to amplify the SCAR marker consisted of 35 cycles each with the following sequence: 30 s at 94 °C, 60 s at 65 °C and 90 s at 72 °C. The amplified bands were analyzed as described for the RAPD technique.

Data analysis

Chi-square analyses and Yates' correction for continuity (Yates, 1934) were used to confirm the mendelian segregation of the resistance phenotypes and the RAPD markers and the linkage between the RAPD markers and the resistance gene.

For the linkage analyses 167 F_2 plants from the cross between Mexico 54 and Ruda were used. The distances between the RAPD markers and the resistance gene were estimated with the program MAPMAKER/EXP, version 3.0

(Lander *et al.*, 1987; Lincoln *et al.*, 1992) with a *lod score* minimum of 3.0.

RESULTS AND DISCUSSION

Disease Inheritance

The segregation ratios of 3:1 in the F_2, 1:1 in the BC_S (backcross to the susceptible parent), 1:0 in the BC_R (backcross to the resistant parent) (Table 1) indicate that resistance of cultivar Mexico 54 to pathotype 63-19 of *P. griseola* is monogenic and dominant. In beans, several workers have demonstrated that the resistance to this pathogen is due to one, two or three dominant or recessive genes. Barros *et al.* (1957) observed that in the majority of the crosses resistance to ALS was recessive and was due to two or three genes, however, in some crosses the resistance was dominant. In crosses between the susceptible cultivars Algarrobo and line 223, and the resistant line 0258, Cardona-Alvarez (1962) observed that resistance to ALS was monogenic and dominant. Singh & Saini (1980) determined that ALS resistance of the *P. coccineus* L. cultivar PLB was due to recessive genes. In Brazil, Santos Filho *et al.* (1976) demonstrated that in the cross involving the resistant cultivar Caraota 260 and the susceptible Venezuela 350, resistance to *P. griseola* was also due to one recessive gene. Sartorato *et al.* (1993) observed that in crosses between the resistant cultivar Cornell 49242 and the susceptible cultivars Rosinha G-2 and Caraota 260, resistance was monogenic and dominant. When cultivar Cornell 49242 was crossed with the resistant cultivar Diacol Nima two dominant resistance genes segregated. Recently, Carvalho *et al.* (1997) and Ferreira (1998) observed that the resistance to ALS was due to one dominant gene in crosses involving lines AND 277 (resistant to pathotype 63-23) and MAR 2 (resistant to pathotype 63-39).

The bean breeding programs in Brazil, until recently, did not consider resistance to ALS as a priority because in the early 80´s the disease usually used to appear at the end of the crop cycle and caused little crop damage. Today, the disease symptoms can be noticed mostly during flowering time and in some cases it can be seen as early as in the primary leaves. In both cases losses can be high. The identification of these genes should contribute to such programs toward the development of new bean cultivars resistant to this disease.

RAPD markers

Three polymorphic DNA bands were detected between the contrasting bulks constructed with the DNA from F_2 plants derived from the cross Mexico 54 x Ruda. These bands were present in all resistant and absent in all susceptible individuals of the bulks. Figure 1 depicts the amplification patterns obtained with primers OPN02 ($^{5'}$ACCAGGGGCA$^{3'}$), OPAC14 ($^{5'}$GTCGGTTGTC$^{3'}$) and OPE04 ($^{5'}$GTGACATGCC$^{3'}$) linked in coupling phase to the resistant gene. In the co-segregation analysis with 167 F_2 plants, these markers were mapped at 5.9, 6.6 and 11.8 cM of the resistance gene (Table 1), with *lod scores* of 25.83, 24.90 and 19.03, respectively.

In common beans, RAPD markers have been used for selecting resistant genotypes to several diseases (Haley *et al.*, 1993; Johnson *et al.*, 1995; Young & Kelly, 1994; Alzate-Marin, 1996). However, molecular markers linked to ALS resistance have been identified only very recently (Carvalho *et al.*, 1997; Ferreira, 1998). *P. griseola* is a pathogen with great genetic variability (Sartorato *et al.*, 1991; Nietsche, 1997) and, probably, with a great number of virulence genes. As a result, it seems reasonable to think that common beans must have resistant genes effective against different pathotypes. Consequently, identification of pathotypes and these genes is extremely important for bean breeding programs.

TABLE 1 - Inheritance and genetic linkage between molecular markers and the angular leaf spot disease resistance gene (R) to pathotype 63-19 of *Phaeoisariopsis griseola* in the cross between bean (*Phaseolus vulgaris*) Mexico 54 (resistant) and Ruda (susceptible) cultivars

Locus	Generation analyzed	Expected Ratio in F_2	Observed ratio	Chi-Square	Probability	Genetic distance[a].
R	F_2	3:1[b]	125:42	0.006	0.90-0.95	-
R	BC_S	1:1[c]	21:25	0.368	0.50-0.70	-
R	BC_R	1:0[d]	29:0	0.00	1,00	-
N 02_{890}	F_2	3:1	124:43	0.04	0.80-0.90	-
AC $14_{2,400}$	F_2	3:1	128:39	0.29	0.50-0.70	-
E 04_{650}	F_2	3:1	125:42	0.005	0.90-0.95	-
R/N 02_{890}	F_2	9:3:3:1[e]	123:7:1:36	118.91	>0.0001	5.9
R/AC $14_{2,400}$	F_2	9:3:3:1	127:3:1:36	128.37	>0.0001	6.6
R/E 04_{650}	F_2	9:3:3:1	116:9:9:33	84.89	>0.0001	11.8

[a]Genetic distances in centiMorgans;
[b]Expected ratio for dominant monogenic inheritance in the F_2 progeny (3 resistant, R_: 1 susceptible, rr);
[c]Expected ratio for dominant monogenic inheritance in the backcross to the susceptible parent (1 resistant, Rr: 1 susceptible, rr);
[d]Expected ratio for dominant monogenic inheritance in the backcross to the resistant parent (1 resistant, R_: 0 susceptible, rr);
[e]Expected ratio for segregation of two independent genes in the F_2 progeny (R_/+:R_/-:rr/+:rr/-).

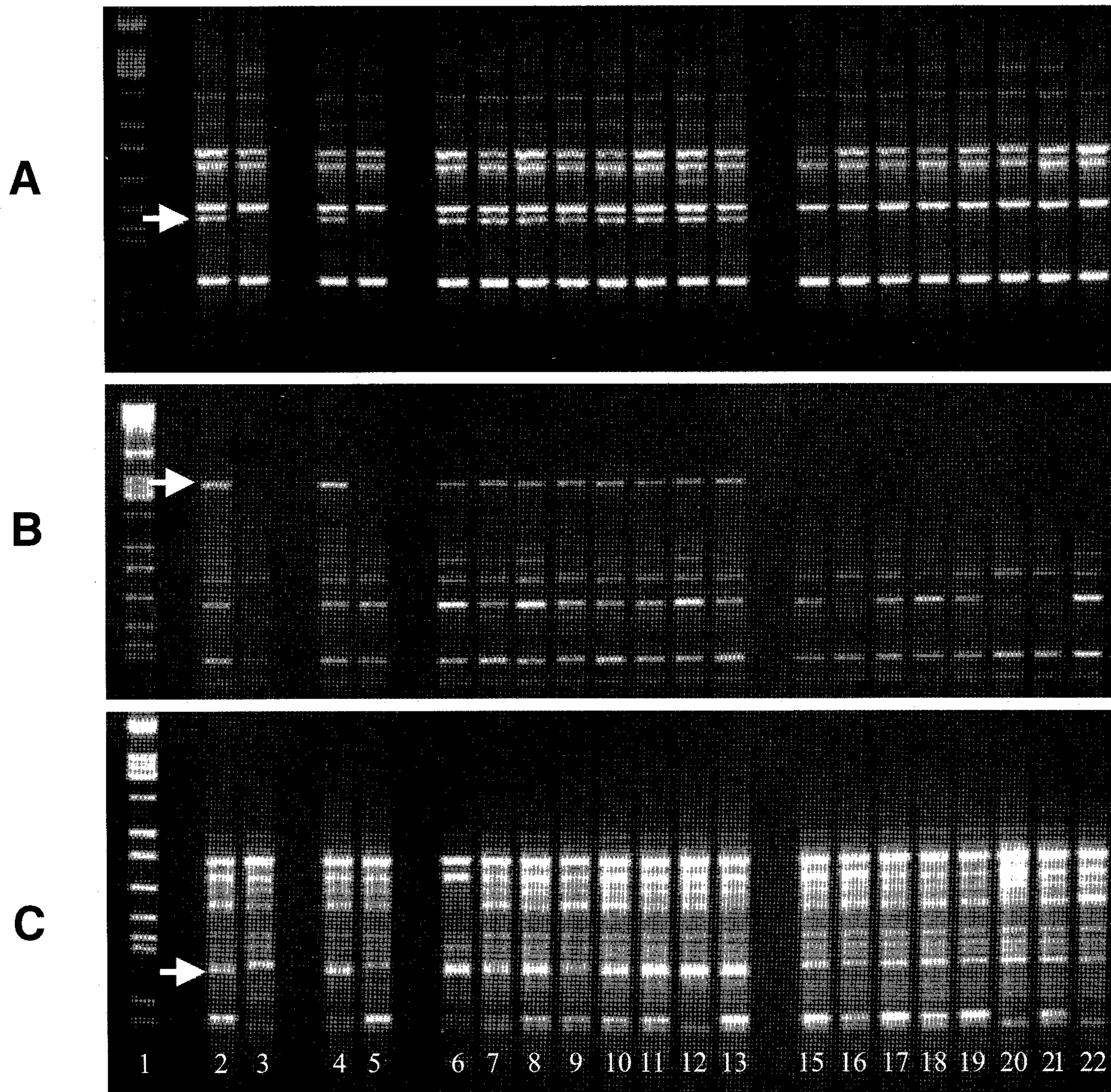

FIG. 1 - DNA amplification products obtained with RAPD primers OPN02 (A), OPAC14 (B), and OPE04 (C) analyzed electrophoretically on agarose gel. Lanes are as follows: 1, lambda phage DNA digested with EcoRI, BamHI and HindIII (size markers); 2, resistant cultivar (Mexico 54); 3, susceptible cultivar (Ruda); 4, resistant bulk; 5, susceptible bulk; 6, 6-13, resistant F_2 plants, and 7, 14-21 susceptible F_2 plants. The arrows indicate the polymorphic DNA bands.

SCAR marker

RAPD markers have been used to monitor disease resistance genes in several plant species, including common beans. However, the usefulness of this type of marker has been questioned (Penner *et al.*, 1993) due to problems related with the reproducibility of the technique in different laboratories. The SCARs represent an interesting alternative to increase reproducibility of RAPD markers (Adam-Blondon *et al.*, 1994).

The DNA fragment corresponding to RAPD marker $N02_{890}$ was cloned and partially sequenced. Two primers of 20 nucleotides each were synthesized based on the sequencing data: SCAR N02 forward - $^{5'}$**ACCAGGGGCA**TTATGAACAG$^{3'}$ and SCAR N02 reverse - $^{5'}$**ACCAGGGGCA**ACATACTATG$^{3'}$. The bold faced nucleotides correspond to the sequence of the original RAPD primer. These two *primers* were tested in the parents, resistant and susceptible bulks and F_2 population of the cross Mexico 54 x Ruda. The polymorphism observed in the amplifications was identical to the one revealed with the corresponding RAPD marker (Figure 2).

LITERATURE CITED

ADAM-BLONDON, A.F., SEVIGNAC, M., BANNEROT,

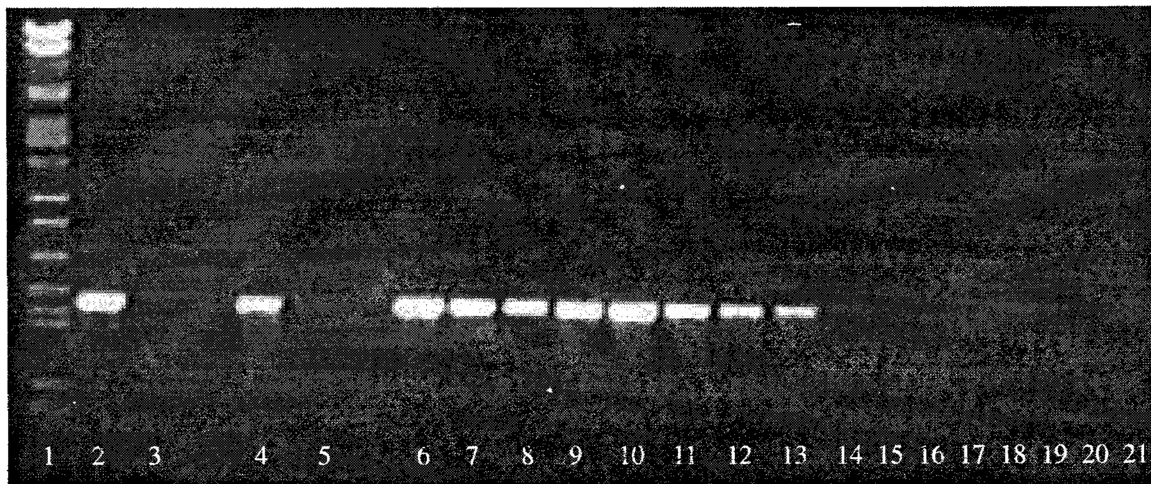

FIG. 2 - DNA amplification products obtained with the OPN02 SCAR primers analyzed electrophoretically on agarose gel. Lanes are as follows: 1, lambda phage DNA digested with *Eco*RI, *Bam*HI and *Hin*d III (size markers); 2, resistant cultivar (Mexico 54); 3, susceptible cultivar (Ruda); 4, resistant bulk; 5, susceptible bulk; 6-13, resistant F_2 plants; 14-21, susceptible F_2 plants.

H. & DRON, M. RAPD and RFLP markers linked to *Are*, a simple dominant gene conferring resistance to *Colletotrichum lindemuthianum*, the causal agent of anthracnose in french bean. Theoretical and Applied Genetics 88:865-870. 1994.

ALZATE-MARIN, A.L. Resistência à antracnose do feijoeiro (*Phaseolus vulgaris* L.): diversidade genética de raças de *Colletotrichum lindemuthianum*, herança da resistência e identificação de marcadores moleculares. (PhD Thesis). Viçosa Universidade Federal de Viçosa. 1996.

BARROS, O., CARDEÑOSA, R. & SKILES, R.L. The severity and control of angular leaf spot of beans in Colombia. Phytopathology 47:3. 1957. (Suplemento).

CARDONA-ALVAREZ, C. Herencia de la resistencia a la mancha angular en frijol. Agronomía Tropical 18:330-331. 1962.

CARVALHO, G.A., NIETSCHE, S., ALZATE-MARIN, A.L., FERREIRA, C.F., PAULA JR., T.J., FALEIRO, F.G., BARROS, E.G. & MOREIRA, M.A. Identificação de marcadores RAPD ligados a genes de resistência à mancha-angular do feijoeiro. Fitopatologia Brasileira 22:255. 1997. (Suplemento).

CIAT (Centro International de Agricultura Tropical). Standard system for the evaluation of bean germplasm. van SCHOONHOVEN, A., PASTOR-CORRALES, M.A. (compilers). Cali, Colombia. 1987.

DOYLE, J.J. & DOYLE, J.L. Isolation of plant DNA from fresh tissue. Focus 12:13-15. 1987.

FERREIRA, C.F. Herança da resistência do feijoeiro à mancha-angular e identificação de marcador RAPD ligado ao gene de resistência. (MS Thesis). Viçosa. Universidade Federal de Viçosa. 1998.

HALEY, S.D., MIKLAS, P.N., BYRUM, J. & KELLY, J.D. Identification of RAPD markers linked to a major rust resistance gene block in common bean. Theoretical and Applied Genetics 86:505-512. 1993.

HALEY, S.D., AFANADOR, L.K. & KELLY, J.D. Identification and application of RAPD marker for the *I* gene (potyvirus resistance) in common bean. Phytopathology 84:157-160. 1994.

JOHNSON, E., MIKLAS, P.N., STAVELY, J.R. & MARTINEZ-CRUZADO, J.C. Coupling- and repulsion-phase RAPDs for marker-assisted selection of PI 181996 rust resistance in common bean. Theoretical and Applied Genetics 90:659-664. 1995.

LANDER, E.S., GREEN, P., ABRAHAMSON, J., BAARLOW, A., DALY, M.J., LINCOLN, S.E. & NEWBURG, L. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181. 1987.

LINCOLN, S., DALY, M. & LANDER, E. Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical Report. 3rd edition. 1992.

MICHELMORE, R.W., PARAN, J. & KESSELI, R.V. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregation populations. Proceedings National Academy Science USA 88:9828-9832. 1991.

MORA-BRENES, B., CHAVES, G.M. & ZAMBOLIM, L. Estimativas de perdas no rendimento do feijoeiro comum (*P. vulgaris* L.) causadas pela mancha angular (*Isariopsis griseola* Sacc.). Fitopatologia Brasileira 8:599. 1983. (Suplemento).

NIETSCHE. S. Identificação de raças de *Phaeoisariopsis griseola* e determinação de fontes de resistência em

Phaseolus vulgaris. (MS Thesis). Viçosa. Universidade Federal de Viçosa. 1997.

PASTOR CORRALES, M.A. & JARA, C.E. 1995. La evolución de *Phaeoisariopsis griseola* con el frijol comun en América Latina. Fitopatologia Colombiana 19:15-24.

PENNER, G.A., BUSH, A., WISE, R., KIM, W., LES DOMIER, KASHA, K., LAROCHE, A., SCOLES, G., MOLNAR, S.J. & FEDAK, G. Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. PCR Methods and Applications 2:341-345. 1993.

SANTOS FILHO, H.P., FERRAZ, S. & VIEIRA, C. Resistência à mancha angular (*Isariopsis griseola* Sacc.) no feijoeiro (*Phaseolus vulgaris* L.). Revista Ceres 23:226-230. 1976.

SARTORATO, A. & RAVA, C.A. Influência da cultivar e do número de inoculações na severidade da mancha angular (*Isariopsis griseola*) e nas perdas na produção do feijoeiro comum (*Phaseolus vulgaris*). Fitopatologia Brasileira 17:247-251. 1992

SARTORATO, A., RAVA, C.A., MENTEN, J.O.M. & BERGAMIN FILHO, A. Resistência vertical do feijoeiro comum (*Phaseolus vulgaris*) a *Isariopsis griseola*. Fitopatologia Brasileira 16:43-46. 1991

SARTORATO, A., ZIMMERMANN, M.J.O., RAVA, C.A. & CARNEIRO, J.E.S. Inheritance of dry bean resistance to *Isariopsis griseola*. Summa Phytopathologica 19:30. 1993. (Suplemento).

SINGH, A.K. & SAINI, S.S. Inheritance of resistance to angular leaf spot (*Isariopsis griseola* Sacc.) in french bean (*Phaseolus vulgaris* L.). Euphytica 29:175-176. 1980.

WILLIAMS, J.G.K., KUBELICK, A.R., LIVAK, K.J., RAFALSKI, J.A. & TINGEY, S.V. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531-6535. 1990.

YATES, F. Contingency tables involving small numbers and the c^2 test. Journal of the Royal Statistic Society 1:217-235. 1934.

YOUNG, R.A. & KELLY, J.D. A RAPD marker for the *Are* anthracnose resistance gene in beans. In: Annual Report of the Bean Improvement Cooperative 37:77-78. 1994.

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