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Introgression of *Co-4*^{2} and *Co-5* anthracnose resistance genes into 'Carioca' Common bean cultivars

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ABSTRACT - *Anthracnose, caused by* Colletotrichum lindemuthianum*, is one of the most destructive diseases to affect the common bean (*Phaseolus vulgaris *L.). Cultivar G 2333 is one of the most resistant cultivars, being the donor parent of genes* Co-4^2 *and* Co-5 *in the pyramiding breeding program of BIOAGRO/UFV. We aimed at: a) the development of resistant homozygous 'carioca-type' common bean lines separately carrying the* Co-4^2 *and* Co-5 *genes derived from G 2333, with the marker assisted selection (MAS), and b) the characterization of the resistant lines with different pathotypes of* C. lindemuthianum*. Thirteen lines of the 'carioca grain type' were selected; ten carrying the* Co-4^2 *and three the* Co-5 *gene, all showing resistance to the anthracnose pathotypes tested in this work. The association of adapted lines with the* Co-4^2 *gene combined with other resistance genes should provide durable resistance to this disease in Brazil and in other parts of the world.*

Key words: *Colletotrichum lindemuthianum*, RAPD molecular markers, resistance, gene pyramidation, *Phaseolus vulgaris* L.

INTRODUCTION

Anthracnose, caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams. - Scrib., is one of the most destructive diseases for common bean (*Phaseolus vulgaris* L.) in tropical and subtropical areas of Latin America, Central and Eastern Africa (Pastor-Corrales 1985). Cultivar G 2333, selected as one of the twelve international differential common bean cultivars for anthracnose, is among the most resistant cultivars to this disease (Pastor-Corrales 1992, Sharma et al. 1999). This cultivar showed resistance to 50 *C. lindemuthianum* pathotypes collected from several regions growing common bean in Brazil (Alzate-Marin and Sartorato 2004). The genetic resistance conferred by G 2333 has also proven to be effective against most *C. lindemuthianum* isolates from South, Central and North America, and India (Balardin and Pastor-Corrales 1990, Araya et al. 1991, Pastor-Corrales et al. 1994, Balardin and Kelly 1998, Sharma et al. 1999). Genetic studies confirmed that G 2333 carries three independently inherited resistance genes: *Co-4*^{2} (an allele of *Co-4*), *Co-5*, and a third poorly characterized gene, tentatively designated *Co-7* (Young et al. 1998).

Given the presence of at least two well-characterized resistance genes (*Co-4*^{2} and *Co-5)* in cultivar G 2333 against important *C. lindemuthianum* pathotypes that occur in Brazil (Alzate-Marin et al. 2001), and the broad resistance spectrum of this cultivar, we selected it as one of the donor parents for our

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common bean breeding program. Besides, *Co-4²* is the most effective anthracnose resistance gene characterized to date and it is the only gene able to overcome pathotype 2047, which was identified in Costa Rica and is one of the most virulent collected so far (Balardin and Kelly 1998).

The use of resistant cultivars has been considered an efficient, safe, and inexpensive method for controlling common bean pathogens. The backcross breeding method is largely used to transfer traits with high heritability, such as disease resistance, to elite genotypes. Molecular fingerprints can be used to aid the selection of individuals that bear the gene of interest and, in addition, possess a high proportion of the recurrent parent's genome (Openshaw et al. 1994).

Objectives of this work were (1) to select 'carioca grain type' common bean lines carrying, separately, genes *Co-4²* and *Co-5* from G 2333 using molecular marker-assisted selection (MAS), (2) to select homozygous common bean lines possessing genes *Co-4²* or *Co-5*, genetically closest to the recurrent parent Rudá, and (3) to characterize these homozygous lines with respect to their resistance against *C. lindemuthianum* pathotypes.

MATERIAL AND METHODS

C. lindemuthianum pathotypes and cultivation procedures

Pathotypes 7, 55, 64, 65, 73, 87, 119, and 453 used in this work are part of a group of 25 pathotypes collected in different regions of Brazil and identified by Rava et al. (1994). The original inocula were provided by CA Rava and A Sartorato (Rice and Bean Research Center - Embrapa, Goiânia, Goiás, Brazil). Isolates of pathotypes 81 and 89 were collected in Viçosa (Minas Gerais, Brazil) and identified in our bean breeding program. The isolate of pathotype 2047 (classified by Balardin et al. 1997) was provided by JB Santos from the Federal University of Lavras and was used for the inoculation of lines with the *Co-4²* gene. The inocula were multiplied by cultivating the fungi for approximately 10 days in sterile medium containing young green common bean pods (Pio Ribeiro and Chaves 1975).

Parents and crosses

Seeds from the cultivars Rudá and G 2333 were provided by Embrapa (Goiânia, Goiás, Brazil) and CIAT (Tropical Agriculture International Center, Cali, Colombia), respectively. Rudá is a commercial Mesoamerican cultivar, indeterminate type, with small 'carioca grain types'. G 2333 is a Mesoamerican cultivar, indeterminate type, with small red seeds. Cultivars Rudá and G 2333 were crossed in the greenhouse under controlled environmental conditions. G 2333 was used as the pollen donor. Backcrosses were made using Rudá as recurrent parent.

DNA extraction, amplification conditions, MAS and genetic distances

Leaf DNA was extracted by a mini-prep procedure based on Doyle and Doyle (1990). DNA amplification reactions were performed according to Williams et al. (1990) while the amplification cycles, product analyses, and band visualization followed Alzate-Marin et al. (2001). Polymorphic RAPD primers between Rudá and G 2333 used to evaluate genetic distances were selected after testing about 200 primers (data not shown).

Plants with the *Co-4²* gene were selected along three backcrossing generations with the aid of RAPD markers $OPH18_{1200C}$ and $OPAS13_{950C}$ (Young et al. 1998, Alzate-Marin et al. 2001) linked to the *Co-4²* gene and also based on symptom evaluation (resistance to *C. lindemuthianum* pathotype 73). Selection of plants genetically closer to the recurrent parent Rudá was based on profiles (fingerprinting) obtained with RAPD markers. In each backcross generation the BC_nF_1 plants were inoculated with spore suspensions of *C. lindemuthianum* (1.2×10^6 spores mL^{-1}) according to Pio Rivero and Chaves (1975). The plants were then incubated for seven days in a mist chamber at 20 - 22 °C and 100% relative humidity. After this period, each plant was scored visually for the disease symptoms by a 1 - 9 scale (Rava et al. 1993) in which 1 was attributed to plants with no visible symptoms and 9 to severely diseased or dead plants. Leaf DNA was extracted from the parents and from the resistant BC_nF_1 plants by a mini-prep procedure based on Doyle and Doyle (1990). According to Williams et al. (1990) different primer sets were used in each backcross cycle for the amplification reactions. Genetic distances and cluster analyses were performed from Euclidean method for binary data (StatSoft Inc 1995). For the analyses, the highest genetic distance was considered to be 100% and the other data points were expressed as a percentage of that distance. Pairwise genetic distances between the recurrent parent and BC_nF_1 plants were analyzed. The plants genetically closest to the recurrent parent in each backcross cycle were chosen for the next backcross.

$BC_3F_{2:3}$ resistant plants of families segregating for one gene (3:1) were identified and selfed. These lines were analyzed again with the RAPD markers $OPH18_{1200C}$ and $OPAS13_{950C}$ to ensure the presence of gene *Co-4²*. Progeny tests with *C. lindemuthianum* pathotype 73 were realized to identify 10 non-segregating lines. These lines were selfed for three consecutive generations.

For the selection of BC_1F_1 plants carrying only the *Co*-5 gene, the absence of RAPD marker $OPH18_{1200C}$ was used as selection criterion. Only one BC_1F_1 plant derived from the cross Rudá *vs* G 2333 did not harbor this marker. This plant generated one BC_1F_2 population that was inoculated with *C. lindemuthianum* pathotype 89. The presence of RAPD marker $OPAB03_{450C}$ linked

to gene *Co-5* (Young et al. 1998) and absence of marker $OPH18_{1200C}$ were tested in this population. $BC_1F_{2:3}$ resistant plants of families segregating for one gene (3:1) were identified and selfed. These lines were again analyzed with RAPD marker $OPAB03_{450C}$ to ensure that the *Co-5* gene was present after selfing. Progeny tests with *C. lindemuthianum* pathotype 89 were conducted to identify the non-segregating lines genetically closest to the recurrent parent Rudá. The homozygous lines were selected and selfed for two consecutive generations.

The RAPD primers used to analyze the BC_3F_6 lines were OPAS-06, OPAS-09, OPAS-12, OPAS-14, OPAU-02, OPAU-05, OPAU11, OPAW-07, OPAW-08, OPAW-15, OPAW-19, OPAX-01, OPAX-06, OPAX-08, OPAX-09, OPAX-10, OPAX-14, OPAX-20, OPAY-10, OPAY-11. To analyze the BC_1F_5 lines the following RAPD primers were used: OPA-20, OPAA-18, OPAK-16, OPAS-06, OPAS-09, OPAS-12, OPAS-14, OPAT-20, OPAU-11, OPAV-11, OPAV-14, OPAW-07, OPAW-19, OPAX-01, OPAX-02, OPAX-05, OPAX-06, OPAX-09, OPAX-11, OPAX-14, OPAY-10, OPAY-11, OPBE-02, OPBE-05, OPBE-20, OPBF-08, OPBF-09, OPBF-10, OPBF-15, OPBF-16, OPBF-19, OPBG-07, OPBG-15, OPBH-01, and OPBH-20. Only strong and reproducible bands were used for the analyses.

Inoculation and disease symptom evaluation

In each backcross generation seeds of the BC_nF_n plants and parents were sown and fourteen days later, the first expanded trifoliate leaf from each plant was inoculated onto the lower and upper leaf surfaces with spore suspensions of *C. lindemuthianum* (1.2×10^6 spores mL^{-1}) pathotypes 73 (lines with gene *Co-4*^{2}) and 89 (lines with gene *Co-5*). Spore suspensions were applied with a De Vilbiss nº 15 apparatus according to Pio Ribeiro and Chaves (1975). The plants were then incubated for seven days in a mist chamber at 20 - 22 ^oC and 100% relative humidity. After this period, each plant was scored visually for the disease symptoms by a 1 - 9 scale (Rava et al. 1993) as described previously. Resistant (R) phenotype was assigned to plants with no or limited symptoms (grades 1 to 3) whereas plants graded 4 or above were considered to be susceptible (S). To avoid cross-contamination, experiments with different pathotypes were conducted in separate chambers.

Characterization of common bean lines with *C. lindemuthianum* pathotypes

To study the anthracnose resistance spectrum of homozygous lines carrying the *Co-4*^{2} or the *Co-5* gene, 12 plants of each line (Table 1), and 12 plants each of cultivars Rudá (susceptible) and G 2333 (resistant), were inoculated with spores of the *C. lindemuthianum* pathotypes 7, 55, 64, 65, 73, 81, 87, 89, 119, and 453. The lines with gene *Co-4*^{2} were also inoculated with spores of pathotype 2047. To avoid cross-contamination, each pathotype was inoculated in a separate chamber. The inoculation conditions and symptom evaluations were the same as described previously. The anthracnose resistance spectra of the new lines were compared with those reported by Rava et al. (1994) and Arruda et al. (2001) for non-adapted black-seeded cultivar Selection 1308 (gene *Co-4*^{2}) and for cultivar TU (gene *Co-5*), respectively (Table 2).

Table 1. Pairwise relative genetic distances (%) between recurrent (Rudá) and donor (G 2333) parents *vs* BC_3F_6 and BC_1F_5 lines carrying the *Co-4*^{2} or *Co-5* genes, respectively

BC_3F_6 lines (*Co-4*^{2})			BC_1F_5 lines (*Co-5*)		
Genotype	**Rudá**	**G 2333**	**Genotype**	**Rudá**	**G 2333**
G 2333	100.00	-	G 2333	100.00	-
341-22-3-19-1-1	0.00[a]	100.00	1-46-1	31.14	68.85
341-22-3-19-1-2	0.00 [a]	100.00	1-46-2	40.98	59.01
341-22-3-19-1-3	0.00 [a]	100.00	1-46-3	37.70	62.29
341-22-3-19-1-4	0.00 [a]	100.00	1-46-5	27.86[a]	72.13
341-22-3-19-1-5	0.00 [a]	100.00	1-46-6	29.50[a]	70.49
341-22-3-19-1-6	0.00 [a]	100.00	1-46-7	27.86[a]	72.13
341-22-3-19-1-7	0.00 [a]	100.00	1-46-8	34.42	65.57
341-22-3-19-11-1	0.00 [a]	100.00			
341-22-3-19-11-2	0.00 [a]	100.00			
341-22-3-19-11-3	0.00 [a]	100.00			

[a]Selected lines

Table 2. Reactions of the "carioca seed type" BC_3F_6 and BC_1F_5 lines, cultivars Selection 1308 and TU to different *Colletotrichum lindemuthianum* pathotypes

Pathotype (Binary nomenclature)	Group/pathotype (Classical nomenclature)	Origin (Country)	Selection 1308 (*Co-4*^{2})[a]	TU (*Co-5*)[b]	BC_3F_6 Lines (*Co-4*^{2})[c]	BC_1F_5 Lines (*Co-5*)[c]
7	DELTA/Delta (Widusa R)	Brazil	RR[d]	RR	RR	RR
55	DELTA/Lambda	Brazil	RR	RR	RR	RR
64	MEXICAN I /Mexican I	Brazil	RR	RR	RR	RR
65	ALFA/Epsilon	Brazil	RR	RR	RR	RR
73	ALPHA/Alpha BR	Brazil	RR	RR	RR	RR
81	ALPHA /Eta	Brazil	RR	RR	RR	RR
87	DELTA/Mu	Brazil	RR	RR	RR	RR
89	ALPHA/Alpha BR	Brazil	RR	RR	RR	RR
119	DELTA/Lambda	Brazil	RR	RR	RR	RR
453	BRASILEIRO I /Zeta	Brazil	RR	RR	RR[e]	RR[e]
2047	-[f]	Costa Rica	RR	-[f]	RR	-[f]

[a]Arruda et al. (2001), [b]Rava et al. (1994), [c]data obtained in this work, [d]RR=homozygous resistant, [e]some plants presented minor symptoms (grades 2 and 3), [f]data not available

RESULTS AND DISCUSSION

Obtaining common bean lines with the *Co-4*^{2} or *Co-5* genes with the aid of molecular markers

Amplification with RAPD markers $OPAS13_{950C}$ (Young et al. 1998) and $OPH18_{1200C}$ (Alzate-Marin et al. 2001) allowed the selection of resistant plants harboring the *Co-4*^{2} gene in generations BC_1F_1, BC_2F_1 and BC_3F_1. To identify lines carrying the *Co-5* gene, the presence of RAPD marker $OPAB03_{450C}$ (Young et al. 1998) and absence of marker $OPH18_{1200C}$ (Alzate-Marin et al. 2001) were determined. Ten non-segregating lines carrying the *Co-4*^{2} gene and seven the *Co-5* gene were obtained. These lines were phenotypically similar to the recurrent parent Rudá and derived from plants 341-22-3-19-1 and 341-22-3-19-11 (gene *Co-4*^{2}), and from plant 1-46 (gene *Co-5*) (Table 1).

Genetic distances

Pairwise genetic distances between recurrent parent Rudá and BC_3F_6 and BC_1F_5 plants with 'carioca grain type' were used to select lines with the *Co-4*^{2} or the *Co-5* anthracnose resistance genes, respectively, genetically similar to the recurrent parent. All BC_3F_6 plants showed genetic distances of zero in relation to Rudá (Table 1). Higher genetic distances between the BC_1F_5 lines and recurrent parent Rudá were observed because only one backcross was conducted for their development. However, three lines phenotypically similar to Rudá with distances of 27.86 and 29.50% were selected among the BC_1F_5 lines (Table 1).

Resistance spectrum

Inoculation of homozygous BC_3F_6 and BC_1F_5 lines with 11 and 10 *C. lindemuthianum* pathotypes, respectively, demonstrated that the new 'carioca grain type' selected lines are resistant to all pathotypes studied (Table 2). This same spectrum was observed for non-adapted common bean black-seeded cultivars Selection 1308 and TU, which possess the same resistance genes *Co-4*^{2} and *Co-5*, respectively (Rava et al. 1994, Arruda et al. 2001).

CONCLUSIONS

In this work we showed the development of 'carioca grain type' common bean lines separately carrying the two major anthracnose resistance genes present in cultivar G 2333, *Co-4*^{2} and *Co-5*, with the aid of RAPD molecular markers. The strategy used - indirect selection with molecular markers linked to the resistance genes and DNA fingerprinting - considerably decreased the time normally required to recover the genome of the recurrent parent Rudá. RAPD markers $OPAS13_{950}$ and $OPH18_{830}$, tightly linked to the *Co-4*^{2} gene, and RAPD marker $OPAB03_{450C}$ linked to the *Co-5* gene, were important tools for the identification and confirmation of the presence of the anthracnose resistance genes in the lines obtained in the backcross program.

The common bean-breeding program of BIOAGRO/UFV now possesses thirteen 'carioca grain type' lines that carry the *Co-4*^{2} or the *Co-5* genes, genetically similar to the 'carioca' cultivar Rudá, with similar resistance spectra to those of cultivars Selection 1308 (*Co-4*^{2}) and TU (*Co-5*). The lines we developed can now be used in common bean programs that aim at pyramiding anthracnose resistance genes and/or as adapted gene sources. As *Co-4*^{2} is the most effective anthracnose resistance gene characterized to date, the adapted cultivars with this gene will

certainly become very important for common bean breeding programs in Brazil. With the aid of RAPD markers $OPAS13_{950}$ and $OPH18_{830}$, the *Co-4*^{2} gene can easily be transferred to different genetic backgrounds without the need of using highly virulent non-endemic *C. lindemuthianum* pathotypes (i.e. 1033, 1545, 1600, or 2047) to confirm the presence of the resistance gene. However, the dependence on a single resistance gene may not be a wise strategy for the long-term control of this disease. Therefore, the use of adapted lines with the *Co-4*^{2} gene associated with other resistance genes (like *Co-2*, *Co-5*, or *Co-6*) to local pathotypes would provide a durable resistance to anthracnose in Brazil and in other parts of the world.

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Introgressão dos genes de resistência à antracnose *Co-4*^{2} e *Co-5* em cultivares de feijoeiro Carioca

RESUMO - *A antracnose, causada pelo fungo* Colletotrichum lindemuthianum, *é uma das doenças mais destrutivas que afetam o feijoeiro comum (*Phaseolus vulgaris *L.). G 2333 está entre os cultivares mais resistentes a ela e é o doador dos genes* Co-4^2 *e* Co-5 *no programa de melhoramento para piramidação de genes do BIOAGRO/UFV. Os objetivos deste trabalho foram: a) usar a seleção assistida por marcadores moleculares (MAS) no desenvolvimento de linhagens resistentes de grãos tipo carioca, carregando separadamente os genes* Co-4^2 *e* Co-5, *e b) caracterizar o espectro de resistência das linhagens a diferentes patótipos de C. lindemuthianum. Treze linhagens com grãos do tipo carioca foram selecionadas; dez carregando o gene* Co-4^2 *e três o gene* Co-5, *todas resistentes aos patótipos testados. A associação de linhagens adaptadas com o gene* Co-4^2 *com outras fontes de resistência à antracnose poderá prover resistência durável a essa doença no Brasil e em outras partes do mundo.*

Palavras-chave: *Colletotrichum lindemuthianum*, marcadores moleculares RAPD, resistência, piramidação de genes, *Phaseolus vulgaris* L.

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