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ARTICLE

Introgression of resistance to pathogens in common bean lines with the aid of molecular markers

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Abstract – *Aiming to incorporate resistance to the anthracnose and angular leaf spot pathogens in the common bean lines BRSMG Talismã, VC8 and VC9, crosses were made between these lines and the line Rudá-R, donor of the alleles Phg1, Co-4, Co-10, which confer resistance to* Pseudocercospora griseola *and* Colletotrichum lindemuthianum*. After the crosses, the backcross populations were obtained, and the RC_1F_1 plants were inoculated with race 65 of* C. lindemuthianum*. The resistant plants were genotyped with the markers SCARH13, SCARY20 and SCARF10, linked to the alleles Phg1, Co-4 and Co-10, respectively. Based on the molecular data, 44 plants were selected. The progenies originating from multiplication of these plants were evaluated over three seasons for grain yield, plant architecture and grain aspects. Based on these considerations and molecular data, 13 promising progenies were selected for developing inbreds.*

Key-words: *Marker-assisted selection,* Phaseolus vulgaris*, anthracnose, angular leaf spot.*

INTRODUCTION

Brazil is the world's largest producer of common bean and, currently, annual yield is around 951 kg ha^{-1} (Conab 2012). This yield is much below crop potential, with diseases being one of the main causes of this situation. Among the fungal diseases of the above ground part, anthracnose and angular leaf spot stand out, which are caused by the pathogens *Colletotrichum lindemuthianum* and *Pseudocercospora griseola*, respectively (Paula Júnior and Zambolim 2006).

In recent years, common bean breeders have made great effort in developing common bean cultivars with resistance to anthracnose and angular leaf spot. In spite of great advances, up to the present time, common bean cultivars of the carioca type (beige with brown stripes) with broad resistance to these pathogens is not available. This is due to their great physiological variability and especially to the great difficulty of associating alleles of resistance to the diverse races of the different pathogens in the same cultivar through conventional methods (Michelmore 1995). In this respect, an alternative that is being used in some breeding programs consists of molecular marker assisted selection (Kelly and Miklas 1998, Lawson et al. 1998).

Disease resistance is not the only trait for improvement in a breeding program. Others, such as yield, plant architecture and grain aspects are of fundamental importance for the success of a common bean cultivar (Cunha et al. 2005, Menezes Júnior et al. 2008, Mendes et al. 2009).

The aim of this study was to obtain progenies of common bean with carioca type grains resistant to anthracnose and to angular leaf spot arising from crosses between the Rudá-R resistance allele donor line and the elite lines VC8 and VC9 and the cultivar BRSMG Talismã, with the assistance of molecular markers.

MATERIAL AND METHODS

Phenotypic and molecular characterization of the parents

Artificial inoculations were made in a greenhouse and in mist chambers of the Plant Science Department of the Federal University of Viçosa (Departamento de Fitotecnia da Universidade Federal de Viçosa - DFT/UFV) and of the Crop and Livestock Applied Biotechnology Institute (Instituto de Biotecnologia Aplicada à Agropecuária -

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BIOAGRO/UFV). Studies related to the use of molecular markers were conducted at the Plant Molecular Genetics I laboratory, located at the facilities of BIOAGRO/UFV.

The Rudá-R line (Ragagnin et al. 2003), the BRSMG Talismã cultivar (Ramalho et al. 2002) and the elite lines VC8 and VC9 (Melo et al. 2006) were used as parents. The Rudá-R parent has carioca type grains (beige with brown stripes) and is a carrier of the alleles for resistance to anthracnose (*Co-4* e *Co-10*) and angular leaf spot (*Phg-1*) (Ragagnin et al. 2003). The cultivar BRSMG Talismã and the lines VC8 and VC9 were selected for showing high productive potential associated with good aspect of the beans.

For phenotypic characterization of the parents in regard to reaction to the anthracnose and angular leaf spot pathogens, 12 plants from each one of the parents and from the Pérola cultivar, used as the susceptible control, were inoculated. For *C. lindemuthianum,* the seeds were sown on plastic trays (60 x 40 x 12 cm) and for *P. griseola* in 2.5 L plastic pots. Races 8, 9, 55, 65, 73, 81, 89 and 453 of *C. lindemuthianum* and races 31-17, 63-15, 63-23, 63-31, 63-39, 63-47 and 63-63 of *P. griseola* were used. A mixture of soil and composted manure in the proportion of 3:1 was used as a substrate, mixed with 5 kg m^{-3} of the fertilizer formulation NPK 08:28:16.

Preparation of the inoculum and the inoculations of *C. lindemuthianum* were carried out according to the adapted methodology of Pio-Ribeiro and Chaves (1975). Inoculations were performed seven days after sowing, dusting a suspension containing 1.2 x 10^6 conidia mL^{-1} on both surfaces of the primary leaves with the aid of a De Vilbiss spray gun no. 15 activated by an electric compressor. Evaluation was made at 10 days after inoculation based on a scoring scale described by Pastor-Corrales (1992), ranging from 1 to 9, in which 1 is attributed to immune plants and 9 to dead plants.

Preparation of the inoculum and the inoculations of *P. griseola* were performed as described by Sartorato and Alzate-Marin (2004). Inoculations were performed after the appearance of the second trifoliate leaf, approximately 15 days after plant emergence, on both surfaces of the first pair of trifoliate leaves, with suspension at the concentration of 2 x 10^4 conidia mL^{-1} with the aid of a De Vilbiss spray gun no. 15 activated by an electric compressor. The severity of the disease was evaluated visually at 20 days after inoculation using a nine degree severity scale proposed by Pastor-Corrales and Jara (1995), with 1 being the score associated with immune plants and 9 with severely attacked plants.

To check for the possibility of use of molecular marker assisted selection in the populations, ten plants from each one of the parents were genotyped, with the markers $SCARY20_{830a}$ (Arruda et al. 2000), $SCARF10_{1050a}$ (Correa et al. 2000) and $SCARH13_{520a}$ (Queiroz et al. 2004).

Collection of leaves and extraction of DNA were performed according to the methodology of Doyle and Doyle (1990). Amplification reactions were made in a total volume of 15 µl, containing Tris-HCl 10 mM (pH 8.3), KCl 50 mM, $MgCl_2$ 2 mM, five picomoles from each specific primer, one unit of the Taq polymerase enzyme and approximately 25 ng of DNA. Amplifications were made in the Perkin-Elmer thermal cycler, model 9600 (Williams et al. 1990), programmed for 35 cycles of 94 ºC for 30 s; 65 ºC (SCARY20 and SCARF10) or 59 ºC (SCARH13) for one minute; 72 ºC for 90 seconds; and a final phase of 72 ºC for 7 minutes. The amplification products were stained with 2.0 µl of type IV stain (0.25% bromophenol blue and 60% glycerol). Subsequently, the products were separated in 1.2% agarose gel containing 0.5 µg ml^{-1} of ethydium bromide, immersed in the SB 1X buffer (sodium hydroxide 10 mM, pH 8.5 adjusted with boric acid) (Brody and Kern 2004). The DNA bands were viewed under ultraviolet light and photo digitized using the Eagle Eye II photodocumentation system.

Obtaining segregating populations and selection strategy

Crosses were made in a greenhouse at the DFT/UFV. Three segregating populations were obtained arising from a backcross cycle (RC_1F_1) involving the Rudá-R line (donor parent) with the recurrent parents BRSMG Talismã (population 410) and the elite lines VC9 (population 411) and VC8 (population 412).

Initially, the segregating populations were inoculated with race 65 of *C. lindemuthianum*, with a view toward selection of resistant plants within each population. It is worthy of note that this race is widely distributed throughout Brazilian territory (Rava et al. 1994) and is a race that currently causes significant losses in the common bean crop. For that reason, RC_1F_1 seeds were sown in a greenhouse using the procedure described for phenotypic characterization of the parents. It is important to highlight that in this step the plants selected were those that did not show any symptom of the disease since the dominant allele *Co-4* confers vertical resistance to this race. The RC_1F_1 plants that showed resistance had leaves collected for purposes of genotyping and were transplanted to pots for later collection of their seeds. Leaf collection, DNA extraction, as well as other steps of amplification of the reactions and of photodocumentation were carried out as already described for molecular characterization of the parents.

Of the plants resistant to race 65 of *C. lindemuthianum,* those with good grain aspect and presence of the marks of the alleles of interest were selected. The seeds of these plants were multiplied and collected following the bulk method within progenies.

Afterwards, the selected progenies were evaluated in the field in the dry and winter crop seasons of 2007 and the dry crop season of 2008. The bulk method was adopted within progenies, with selection for grain aspect during growing of the progenies. The experiments were conducted at the experimental station of Coimbra, MG, situated at 690 m altitude, 20° 45' S latitude and 42° 51' W longitude. A 7x7 triple lattice design was used with plots of 2 two-meter rows spaced at 50 cm, with density of 15 seeds per meter. These experiments consisted of selected progenies and the controls Rudá-R, VC8, VC9, BRSMG Talismã and BRSMG Majestoso. In addition to grain yield, plant architecture and grain aspect were evaluated. For evaluation of plant architecture, the modified scoring scale of Collicchio et al. (1997) was used, ranging from 1 to 5, with score 1 being attributed to plants with upright growth habit and 5 to prostrate plants. For evaluation of grain aspect, a 1 to 5 scoring scale was used (Marques Júnior et al. 1997). According to this scale, the lower the score, the better the aspect of carioca type grains; i.e., cream color of the seed coat with light brown stripes.

RESULTS AND DISCUSSION

Phenotypic and molecular characterization of the parents

The severity of the symptoms caused by *C. lindemuthianum* and *P. griseola* in the parents is shown in Table 1. Observe that the Rudá-R line proved to be resistant to all the races of *C. lindemuthianum* used in the inoculations and it was susceptible only to races 63-15, 63-39, 63-47 and 63-63 of *P. griseola*. These results indicate the potential of the Rudá-R line as parent in common bean breeding programs with a view toward resistance to anthracnose and angular leaf spot. Nevertheless, it is fitting to note that for the races of *P. griseola* in which the Rudá-R line was susceptible, the parents VC8 and VC9 were complementary. Thus, crosses between the parents VC8, VC9 and BRSMG Talismã with the Rudá-R line are promising with a view toward developing potential lines with carioca type grains and with broad resistance to the anthracnose and angular leaf spot pathogens.

As expected, the Rudá-R line showed amplification for the three pairs of SCAR primers used (Table 2). Sanglard et al. (2007) and Melo et al. (2008) also obtained this same result with the Rudá-R line. Molecular characterization of the recurrent parents shows the possibility of monitoring of most of the resistance alleles in the populations under selection, with the exception of lines BRSMG Talismã and VC9. The former was not polymorphic for the marker SCARH13, since it also showed amplification for this marker. This result contradicts the result found by Melo et al. (2008) in which this cultivar did not show the band in question and corroborates the result of Sanglard et al. (2007). The VC9 line showed amplification in reference to the primer SCARY20, which marks the allele *Co-4* and, therefore, does not allow the monitoring of this allele in the population arising from this parent. However, this result is a false positive, considering that this line showed susceptibility to race 65 (Table 1) and therefore does not have the *Co-4* allele. It is known that this line has the *Co-10* allele arising from the cultivar Ouro Negro

Table 1. Mean severity of anthracnose and angular leaf spot in common bean genotypes in relation to different races of *Colletotrichum lindemuthianum* and *Pseudocercopora griseola*

Genotypes	**Races of *C. lindemuthianum***							
	8	9	55	65	73	81	89	453
Rudá-R	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
VC 8	7.7	8.1	6.6	8.7	7.7	8.0	9.0	8.4
VC 9	1.0	1.0	1.0	8.6	1.0	1.0	1.0	1.0
BRSMG Talismã	1.0	1.8	1.0	1.0	1.0	1.8	1.0	7.0
Pérola	7.6	8.5	6.0	9.0	8.2	9.0	9.0	7.5
Genotypes	**Races of *P. griseola***							
	31-17	**63-15**	**63-23**	**63-31**	63-39	63-47	63-63	
Rudá-R	1.0	8.0	1.0	1.0	8.2	8.0	9.0	
VC 8	1.0	1.0	1.0	1.0	1.2	1.0	1.0	
VC 9	6.0	1.0	1.0	4.8	2.5	4.8	2.4	
BRSMG Talismã	1.0	5.8	7.2	4.0	5.5	5.8	7.0	
Pérola	6.0	8.0	3.0	7.0	8.4	9.0	9.0	

Mean severity of anthracnose and angular leaf spot according to the scale described by Pastor-Corrales (1992) and Pastor-Corrales and Jara (1995), respectively. Genotypes with mean values greater than 3.0 were considered susceptible. The Perola cultivar was used as a control.

Table 2. Phenotypic characterization (reaction to race 65 of *Colletotrichum lindemuthianum*) and molecular characterization of 44 common bean progenies and their parents

Progenies	Identification	Race 65	Markers			Progenies	Identification	Race 65	Markers		
			SCARY20 (*Co-4*)[1]	SCARF10 (*Co-10*)[1]	SCARH13 (*Phg1*)[1]				SCARY20 (*Co-4*)[1]	SCARF10 (*Co-10*)[1]	SCARH13 (*Phg1*)[1]
1	410F2-1	R	+[2]	+	*	25	411F2-126	R	*	+	+
2	410F2-4	R	-	+	*	26	411F2-139	R	*	+	+
3	410F2-50	R	+	+	*	27	411F2-145	R	*	+	+
4	410F2-59	R	+	+	*	28	411F2-150	R	*	+	+
5	410F2-90	R	+	+	*	29	411F2-151	R	*	+	+
6	410F2-91	R	+	+	*	30	412F2-1	R	+	+	+
7	410F2-115	R	+	+	*	31	412F2-4	R	+	+	+
8	410F2-125	R	+	+	*	32	412F2-13	R	+	+	-
9	410F2-135	R	+	-	*	33	412F2-14	R	+	+	+
10	410F2-155	R	+	+	*	34	412F2-16	R	+	+	-
11	410F2-161	R	+	+	*	35	412F2-27	R	+	+	+
12	410F2-165	R	+	+	*	36	412F2-31	R	+	+	-
13	411F2-6	R	*	+	+	37	412F2-37	R	+	+	-
14	411F2-35	R	*	+	+	38	412F2-41	R	+	+	+
15	411F2-49	R	*	+	+	39	412F2-45	R	+	-	+
16	411F2-58	R	*	+	+	40	412F2-55	R	+	+	+
17	411F2-67	R	*	+	+	41	412F2-61	R	+	+	-
18	411F2-69	R	*	+	+	42	412F2-65	R	+	+	+
19	411F2-71	R	*	+	+	43	412F2-71	R	+	+	+
20	411F2-76	R	*	+	+	44	412F2-86	R	+	+	+
21	411F2-85	R	*	+	+	Parent	Talismã	R	-	-	+
22	411F2-96	R	*	+	+	Parent	VC8	S	-	-	-
23	411F2-119	R	*	+	+	Parent	VC9	S	+	-	-
24	411F2-120	R	*	+	+	Parent	Rudá-R	R	+	+	+

[1] Resistance alleles linked to the respective markers; R – resistant / S – susceptible; [2] + = presence of the mark / - = absence of the mark; and * non-polymorphic parents.

(Melo et al. 2008). This fact is confirmed by the phenotypic characterization in which VC9 proved to be resistant to race 453 of *C. lindemuthianum* since this dominant allele confers vertical resistance to this race (Table 1).

Obtaining segregating populations and selection strategy

Of the 350 RC_1F_1 plants from the three segregating populations initially inoculated with race 65 of *C. lindemuthianum*, 138 showed resistance, with 46 plants from the cross BRSMG Talismã/Rudá-R//BRSMG Talismã (population 410), 65 from the cross VC9/Rudá-R//VC9 (population 411) and 27 from the cross VC8/Rudá-R//VC8 (population 412). The results from genotyping of the 138 resistant plants with the markers SCARH13, SCARY20 and SCARF10, according to the possibility of monitoring in each population are shown in Table 2. Considering the results of genotyping and the aspect of the grains from each plant, 44 were selected, which gave rise to the progenies subjected to field testing (Table 2).

Of the 44 plants selected, only eight (2, 9, 32, 34, 36, 37, 39 and 41) did not show all the possible marks (Table 2). Nevertheless, these plants were maintained due to their resistance to race 65 of *C. lindemuthianum* and to their excellent grain aspect. Only one of the plants resistant to race 65 under inoculation did not show the mark referring to the SCARY20 marker. This result shows that in addition to being very efficient, indirect selection with the use of good markers is viable.

As observed in joint analysis of variance, in reference to evaluation of the progenies for the yield, grain aspect and plant architecture traits, a significant difference was detected among Progenies ($P < 0.05$) for all the traits evaluated. For yield and grain aspect, significant effects were not observed for the Progenies source of variation within populations. Nevertheless, a significant difference was observed among populations ($P < 0.01$) (Table 3).

In most cases, the Progeny x Environment (P x A) interactions and their breakdowns were significant ($P < 0.01$) for yield and grain aspect (Table 3). Reports of significant interaction between genotypes x environments for yield in common bean are frequent in the literature (Carneiro et al. 2002, Ribeiro et al. 2003, Silva et al. 2004, Farinelli and Lemos 2010). Nevertheless, the same is not observed for grain aspect. This interaction possibly occurred due to selection for grain aspect in the progenies from one crop season to another. Nevertheless, even without carrying out selection for grain aspect, Ribeiro et al. (2004) observed a differentiated effect of the environment on grain coloring of carioca type beans. For plant architecture, significance was not observed for the interactions. This fact indicates consistent behavior of the progenies in regard to this trait. However, it is worth noting that this characteristic was evaluated in only two years and in the same crop season (the dry crop season).

Evaluating the behavior of the progenies of the three populations (Table 4), population 412 stood out in regard to the three traits evaluated. Among the 15 most productive progenies, this population contributed to six of them. Nevertheless, it was in the grain aspect and plant architecture traits that this population most stood out, exhibiting 13 and 10 progenies among the 15 with best performance, respectively. This possibly arises from the fact that one of the parents of this population, line VC8, generally presented the best performance in relation to the three traits of interest (Table 4). In addition, it is fitting to note that this parent is the one which best complements the donor parent Rudá-R in relation to the alleles that confer resistance to the anthracnose and angular leaf spot pathogens (Table 1). These

Table 3. Summary of analyses of variance of grain yield (kg ha^{-1}), scores of commercial grain aspect (1 to 5) and plant architecture (1 to 5) in reference to 44 common bean progenies evaluated in the dry/winter crop seasons of 2007 and dry crop season of 2008

SV	df	MS		MS	
		Yield	Architecture	df	Grain Aspect
Environments (A)	2 (1)[1]	4492025.50**	9.55**	2	5.27**
Treatments (T)	48 (48)	689425.32*	0.37**	48	0.85**
Progenies (P)	43 (43)	669997.29*	0.39**	43	0.58**
Population 410	11 (11)	614519.41	0.28**	11	0.43
Population 411	16 (16)	410899.81	0.28**	16	0.28
Population 412	14 (14)	159575.43	0.18**	14	0.29
Among Populations	2 (2)	6620858.42**	3.32**	2	5.89**
Controls (Te)	4 (4)	1014002.30	0.31**	4	3.85**
F *vs* Te	1 (1)	226522.03	0.01	1	0.46
T x A	96 (48)	462506.05**	0.06	96	0.27**
P x A	86 (43)	472969.81**	0.06	86	0.28**
Population 410 x A	22 (11)	244284.11*	0.05	22	0.19*
Population 411 x A	32 (16)	280937.64**	0.05	32	0.15
Population 412 x A	28 (14)	389135.23**	0.07	28	0.26**
Among Populations x A	4 (2)	3853840.58**	0.13	4	1.96**
Te x A	8 (4)	451393.16**	0.01	8	0.24*
P *vs* Te x A	2 (1)	57016.03	0.12	2	0.03
Mean Error	234 (156)	146166.70	0.05	288	0.12
CV (%)		11.78	4.37		11.23
Mean of Progenies		3339.49	3.18		2.66
Mean of Controls		3264.62	3.20		2.77

[1] Values between parentheses refer to degrees of freedom for plant architecture.
** and * significant by the F test at 1 and 5% probability, respectively.

Table 4. Mean values of yield (kg ha^{-1}), grain aspect (scores from 1 to 5[1]) and plant architecture (scores from 1 to 5[2]) and number of common bean progenies per population among the 15 with best performance (NF15+)

	Grain Yield			Grain Aspect			Plant Architecture		
	Mean	NF15+	LI – LS	Mean	NF15+	LI – LS	Mean	NF15+	LI – LS
Progenies	3339.49			2.66			3.18		
Population 410	3041.13	0	2709.79-3477.35	2.72	1	2.33-3.17	3.23	2	2.94-3.67
Population 411	3443.02	9	2784.85-3400.18	2.82	1	2.39-3.17	3.33	3	2.93-3.62
Population 412	3460.85	6	3035.46-3821.53	2.43	13	2.06-2.72	2.97	10	2.71-3.30
BRSMG Talismã*	3073.86			2.61			3.42		
VC8*	3687.29			2.06			2.91		
VC9*	3396.01			2.78			3.43		
Rudá-R*	2806.95			3.83			3.18		
BRSMG Majestoso*	3358.97			2.56			3.07		

[1] Scoring scale according to Marques Júnior et al. (1997); [2] Modified scoring scale according to Collicchio et al. (1997).
* Controls.
LI = lower limit; and LS = upper limit.

results indicate the efficiency of the selection strategy and obtaining segregating populations using phenotypic and genotypic characterization.

Of the 44 progenies evaluated, 13 proved to be promising (4, 6, 11, 12, 13, 19, 22, 24, 30, 31, 38, 40 and 44) for development of lines because, in addition to standing out in regard to traits of interest, they arise from plants resistant to race 65 of *C. lindemuthianum* and are carriers of molecular markers associated with alleles *Phg1, Co-4* and *Co-10.* It is fitting to note that the progenies 2, 9, 32, 34, 36, 37, 39 and 41, originating from the eight plants that did not contain all the marks (Table 2) are not among the 13 most promising. These results corroborate the efficiency of the selection strategy and obtaining segregating populations using phenotypic and genotypic characterization.

CONCLUSIONS

Thirteen progenies of common bean arising from crosses between the Rudá-R resistance allele donor line and the lines VC8 and VC9 and the cultivar BRSMG Talismã were selected, with potential for developing carioca type grains, with high production potential and resistance to anthracnose and angular leaf spot.

Introgressão de resistência a patógenos em linhagens de feijoeiro com auxílio de marcadores moleculares

Resumo – *Visando incorporar resistência aos patógenos da antracnose e da mancha-angular nas linhagens VC8 e VC9 e na cultivar BRSMG Talismã, foram realizados cruzamentos destas com a linhagem Rudá-R, doadora dos alelos Phg1, Co-4, Co-10, que conferem resistência* à Pseudocercospora griseola *e* Colletotrichum lindemuthianum*. Após a realização dos cruzamentos e obtenção das populações de retrocruzamentos, as plantas RC_1F_1 foram inoculadas com a raça 65 de* C. lindemuthianum*. As resistentes foram genotipadas com os marcadores SCARH13, SCARY20 e SCARF10, ligados aos alelos Phg1, Co-4 e Co-10, respectivamente. Com base nos dados moleculares foram selecionadas 44 plantas. As progênies provenientes da multiplicação destas plantas foram avaliadas em três safras quanto* à *produtividade de grãos, arquitetura de planta e aspecto dos grãos. Levando-se em conta estas avaliações e os dados moleculares, foram selecionadas 13 progênies promissoras para a extração de linhagens.*

Palavras-chave: *Seleção assistida por marcadores,* Phaseolus vulgaris*, antracnose, mancha-angular.*

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