Identification and inheritance of an Rdc *gene resistance to soybean stem canker* (Diaporthe phaseolorum *var.* caulivora)

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Identification and inheritance of an *Rdc* gene resistance to soybean stem canker (*Diaporthe phaseolorum* var. *caulivora*)

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Abstract Soybean stem canker (SSC) is caused by two varieties of the fungus *Diaporthe phaseolorum*: var. *meridionalis* (*Dpm*) and var. *caulivora* (*Dpc*). The objective was to identify and characterize the mode of inheritance of *Rdc* genes through a classical Mendelian analysis. Resistant (R) and susceptible (S) genotypes were used to make 288 RxS and 132 RxR crosses, including their reciprocals. Segregating F_2 generations were obtained by self-fertilization of the respective F_1. The incorporation of codominant molecular markers (Single Nucleotide Polymorphism, SNP) allowed the molecular validation of 48.75% of F_1 heterozygous individuals. Parents (R and S), F_1 individuals, and $F_{2:3}$ families (Progeny Test, PT) from COD 1–258-2 population were inoculated with an isolate of *Dpc* (*Dpc*16), previously identified morphologically and molecularly. The assay showed 21 $F_{2:3}$ families categorized as R, 42 segregated R and S, and 11 as S. Particularly, this F_3 population showed 466 individuals R and 274 S. The chi-square goodness of fit test verified that phenotypic segregation for individual plants in F_3 adjusted to a 5:3 ratio (R:S) and the PT results corresponded to the genotypic ratios (1RR: 2Rr: 1rr) of F_2 individuals. Results allowed the identification of a major resistance gene of simple Mendelian inheritance to SSC that was named *Rdc1*. Also,

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independent segregation 9:3:3:1 was verified between this *Rdc1* gene and the gene that regulates flower colour, a typical phenotypic marker in soybean. Based on literature review, this is the first report on resistance genes (*Rdc*) identified for SSC caused by *Dpc*.

Keywords Soybean stem canker · *Diaporthe phaseolorum* var. *caulivora* · F_1 validation by SNP · Progeny test · Inheritance of *Rdc1*

Soybean stem canker (SSC) is caused by two varieties of the fungus *Diaporthe phaseolorum*: var. *meridionalis* (*Dpm*) and var. *caulivora* (*Dpc*) (Fernández and Hanlin 1996; Pioli et al. 2001). In soybean (*Glycine max*), breeding for disease resistance has contributed to effective management of many important diseases. Four dominant resistance genes of Mendelian inheritance against soybean stem canker (SSC) were identified in the 1980s and 1990s; *Rdc1* and *Rdc2* genes in Tracy M cultivar (cv); *Rdc3* in Crockett cv. and *Rdc4* in the genetic background of both Dowling and Hutcheson cvs. (Kilen and Hartwig 1987; Bowers et al. 1993; Tyler 1996). Simultaneously, the use of *forma specialis* (Morgan-Jones 1989) or *variety* (Fernández and Hanlin 1996) was proposed to resolve the taxonomic controversy between the northern (*Dpc*) and southern stem canker (*Dpm*) in the United States of America (USA). The four *Rdc* genes described for SSC in Tracy M, Crockett, Dowling and Hutcheson were renamed as *Rdm* because the corresponding pathogenicity tests and inheritance analysis had been made with *Dpm* isolates (Pioli et al. 2003). Later, Chiesa et al. (2009) identified a new gene, the *Rdm5*, linked to *Rdm4* in Hutcheson cv., located at the *Rdm4–5* locus. Also, *Rdm* (*Rdm1–5*) genes were not effective against SSC caused by *Dpc* (Pioli et al. 2003). Furthermore, the selection pressure given by the incorporation of *Rdm* genes for resistance to SSC caused by *Dpm* in the soybean producing area, promoted the expansion of the SSC disease caused by *Dpc* in Argentina (Pioli et al. 2002; Grijalba and Guillin 2007; Benavidez et al. 2010). Consequently, the SSC by *Dpc* gradually became one of the most important soybean diseases, because *Rdc* resistance genes had not been identified in the soybean germplasm and hence were not available for breeding programs.

The objective of the current was to identify and characterize the inheritance of *Rdc* genes for resistance to SSC-*Dpc* through classical Mendelian analysis with assistance of specific molecular makers. For achieving this objective, twenty-four soybean genotypes that expressed a differential reaction of resistance (R) or susceptibility (S) were selected as parents in different crosses from 137 soybean genotypes and 405 interactions (Pioli et al. 2003; Benavidez et al. 2010) (Table 1 of Supplementary material). Parental genotypes were grown in a greenhouse during September to April 2013/14 and 2014/15, in Campo Experimental Villarino (Zavalla province, 33°01′00″S 60°53′00″W). Crosses were performed among discrepant genotypes with respect to their resistance/susceptibility reaction to SSC by *Dpc*. Several phenotypic makers (form and size of leaf; pod, flower and pubescence color and tegument brightness) were observed and registered during the development of parent genotypes and respective F_1 individuals and segregating F_2 generations.

Two hundred eighty-eight (288) combinations from RxS parents (including reciprocal crosses, SxR) and 132 from RxR parents and their reciprocal, were performed. Hybrid seeds obtained from 79 fertile and effective combinations (60 RxS and 19 RxR), were sown in a greenhouse during September to April 2015/16 in Campo Experimental Villarino. When F_1 individuals from both cross types (RxS and RxR) expanded their second trifoliate leaf, eight discs of healthy leaf tissue were taken from each F_1 plant lyophilized and stored at −80 °C until molecular characterization. Co-dominant molecular markers (Single Nucleotide Polymorphism, SNP) were used to validate the heterozygous identity (Yoon et al. 2007). Seeds F_2 (from the cross COD 1-258-2): Ge(13) resistant x Ge(4) susceptible) were planted in a greenhouse during September to April 2016/17 in Campo Experimental Villarino, to advance the segregating populations. $F_{2:3}$ families, which included 10 F_3 individuals derived from the same F_2 plant, were sown in a greenhouse during 2017 in Campo Experimental Villarino and developed for phenotypic evaluation.

The genotype of each F_2 individual was inferred by the phenotypic characterization (pathogenic reaction) of early F_3 segregating generations and their respective $F_{2:3}$ families (Progeny Test, PT) (Allard 1956). Parents (R and S), five F_1 individuals and families $F_{2:3}$ were inoculated with an isolate of *Dpc16* (Esperanza, Santa Fe, Argentina), previously selected from inoculation trials (Pioli et al. 2003), whose identity was molecularly revalidated by Hernández et al. (2015). At the fully expanded trifoliate leaf stage, seedling hypocotyls were wounded

by cutting a thin portion of the external cellular layer of the stem with a sterile scalpel. The cut was made parallel to the hypocotyls axis, from top to bottom, and the bottom part of the sliced portion remained attached to the stem. A portion of approximately 1.5 × 1.5 mm of mycelium was inserted into the wound and immediately covered with Vaseline® to avoid dehydration. All inoculations were conducted with the same technique. Seedlings were kept in high relative humidity (90–100%) during the first 72 h after inoculation by covering them with a transparent polyethylene tent. Moreover, plants without fungal mycelium were included as experimental control. Each plant was evaluated as (0, 0.3, 0.6, 1) according to a severity scale (Pioli et al. 2003) adapted by Chiesa et al. (2009) (Fig. 1 of Supplementary material). This inoculation technique allowed clear differentiations between resistant from susceptible parents in several studies (Benavidez et al. 2010; Chiesa et al. 2009) and was less aggressive than the toothpick technique, according to other reports (Scandiani et al. 2011; Campbell et al. 2017).

SSC progress was registered from 7 to 56 days post inoculation (dpi) every 7 days. An individual was considered as resistant (R) when at 56 dpi, it showed no symptoms or 0.3 level in the severity diagrammatic scale. Plants with 0.6 to 1 values of severity were considered susceptible (S) (Table 2 of Supplementary material). Phenotypic characterization analysis of F_3 individuals and $F_{2:3}$ families were based on the same criteria. Data were analyzed through the non-parametric test of Chi-Square (χ^2) to estimate goodness of fit to hypothesized ratios according to Bowers et al. (1993) and Tyler (1996). Thus, genotypic frequency of F_2 was also validated by the phenotypic response of F_2 individuals, when they were inoculated with the same *Dpc16* isolate. Also, independent inheritance between the new gene that confers resistance to SSC by *Dpc*, identified in this research, and the known morphological marker *flower color* (**W1** purple dominant / **w1** white recessive) was tested.

From different SxR and RxR and reciprocal combinations, 875 crosses were obtained (Table 1). Effective crosses were verified by the morphologic and structural markers used as control during and after emasculation process, according to Johnson and Bernard (1962). Moreover, those effective pods that completed their development and produced F_1 seeds were considered fertile**.** From a total of 875 hybridizations (RxS and RxR), 312 (35.66%) were effective and fertile crosses (Table 1).

When both cycles were considered (2013/14 and 2014/15), 38.49 (97/252) and 47.06% (120/255) of the RxS crosses were effective and produced F_1 seeds, respectively. Otherwise, from the comparison within the same reproductive cycle (2014/15), only 25.82% (95/368) of RxR crosses were effective and produced F_1 seeds (Table 1). Even though the hybridizations derived from RxS crosses were more effective and fertile than RxR ($\chi^2 = 10.83$; $p < 0.001$), the mean number of seeds per pod registered in both type of crosses (RxS and RxR) was one to three seeds (Peruzzo et al. 2017).

Incorporation of codominant molecular markers (Single Nucleotide Polymorphism, SNP) allowed detection of polymorphisms between differential parents and validation by molecular techniques the F_1 heterozygous individuals. Among the 160 SNP molecular markers analyzed in this study, 142 (88.75%) were registered as polymorphic for each parent couple hybridized. Molecular characterization allowed validation as heterozygous 78 F_1 plants, representing 48.75% from a total of 160 F_1 plants tested.

Phenotypic reaction of the parents to SSC by *Dpc*, evaluated during 56 dpi, showed that Ge(13)-R and Ge(4)-S registered the following proportions: 90 % healthy resistant plants (H/RP): 10 % dead susceptible plants (D/

Table 1 Number of crosses (RxS, RxR and reciprocals) performed in 2013/14 and 2014/15, and proportion of effective and fertile crosses according to morphologic and structural controls

	RxS		RxR		Total
	Effective crosses proportion	Effective reciprocals	Effective crosses proportion	Effective reciprocals	
Cycle 2013/14	43/105	54/147	–	–	97/252
Cycle 2014/15	56/124	64/131	62/229	33/139	215/623
Total	99/229	118/278	62/229	33/139	312/875

Table 2 Phenotypic reaction and genotypic characterization of F_1, F_2, $F_{2:3}$ and F_3 segregating populations obtained from the cross between Ge(13) resistant and Ge(4) susceptible soybean genotypes (COD 1–258-2), in the specific interaction with a *Diaporthe phaseolorum* var. *caulivora* isolate (*Dpc*16)

Parents and progenies	Number of individuals or families inoculated	Hypothesis	Expected	Disease reaction (number of observed plants or families)			χ^2 L[g]	p[i]
				R[f]	Seg	S		
Ge(13)	10[a]							
Ge(4)	10[a]							
F_2COD 258–2	32[a]	3:1[c]	24:8	26		6	0.67 ns[h]	0.41
$F_{2:3}$ COD 1–258-2	74[b]	1:2:1[d]	18.5:37:18.5	21	42	11	4.05 ns	0.13
F_3	740[a]	5:3[e]	462.5:277.5	466		274	0.07 ns	0.79

[a] Number of plants

[b] Number of families; ten inoculated plants per $F_{2:3}$ family

[c] Phenotypic frequency in F_2 population

[d] Genotypic frequency in F_2 population

[e] Phenotypic frequency in F_3 population

[f] R = resistant, Seg = segregating, S = susceptible

[g] Chi-squared calculated based on the genotypic and phenotypic segregation

[h] ns: no significant difference between observed and expected values ($p \leq 0.05$)

[i] Probability of find a value $>\chi^2$ L.

Reaction to SSC-*Dpc* was measured at 56 days post-inoculation

SP); and 20 % H/RP: 80 % D/SP, respectively. Meanwhile, the inoculated heterozygous F_1 and control plants showed no symptoms of SSC-*Dpc*. The Progeny Test from the COD 1–258-2 population showed: 21 $F_{2:3}$ families categorized as R; 42 families R / S categorized as segregating $F_{2:3}$; and 11 $F_{2:3}$ families categorized as S (Table 2 and Table 3 of Supplementary material). The χ^2 value indicates the existence of at least one resistance *Rdc* gene in the soybean germplasm evaluated. Phenotypic segregation of the complete F_3 generation, from the same COD 1–258-2 population, was also analyzed and showed 466 H/RP (resistant) and 274 D/SP (susceptible) (Table 4 of Supplementary material). This population adjusted accurately to the phenotypic segregation 5 H/RP:3 D/SP expected for the inheritance of one gene with complete dominance in the F_3 generation (Table 2). In addition, phenotypic characterization of all genotypes derived from the Ge(13) x Ge(4) cross against SSC-*Dpc* (parents, F_1 individuals, complete F_3 and $F_{2:3}$), and the severity values of SSC registered on a F_2 population (from the same cross) inoculated in the same conditions, confirmed that there was at least one resistance *Rdc* gene in the soybean germplasm evaluated (Table 2).

Only two characters regulated by major genes with Mendelian inheritance (R to SSC-*Dpc* and flower color) could distinguish both parents; Ge(13) is R to SSC-*Dpc* (*Rdc1* gene) and has white flowers (*w1* gene); meanwhile Ge(4) is S to SSC-*Dpc* (*rdc1* gene) and has purple

Table 3 Analysis of segregation of flower color and resistance to SSC caused by *Dpc* characters in the F_2 population (COD 1–258-2, Ge(13) x Ge(4))

F_2 Phenotype	Observed	Expected	(O - E)	$(O - E)^2$	$(O - E)^2/E$
RW1	48	41.6	6.375	40.641	0.9764
Rw1	15	13.9	1.125	1.266	0.0912
rW1	9	13.9	−4.875	23.766	1.7128
rw1	2	4.6	−2.625	6.891	1.4899
Total	74	74	0		$\chi^2_{obs} = 4.270$

flowers (*W1* gene). The joint analysis of the phenotypic marker (flower color) registered on 74 F_2 plants and the individual reaction to SSC-*Dpc* inferred for each these same F_2 plants through $F_{2:3}$ progenies, allowed established that the genes ***Rdc1*/*rdc1*** and ***W1/w1*** are independent and not located on the same chromosome 13, or they are located on the same chromosome 13 but at a distance equal or greater than 50 cM (Table 3).

Until now, the resistance genes to SSC-*Dpc* and consequently their inheritance mode had not been identified (Sun et al. 2012). Thus, the isolation of *Dpc* from different soybean producing agro-ecosystems and selection of differential genotypes made from a wide genetic variability source within the soybean germplasm (Pioli et al. 2003; Benavidez et al. 2010; Peruzzo et al. 2018) allowed to obtain the resistant and susceptible parents and one *Dpc* isolate to be included in this study. Thus, selection of 12 R and S parents was made based on 651 diverse soybean-*Dpc* interactions. A total of 420 (288 + 132) combinations were made; which achieved a total of 875 crosses, although only 312 (35.66%) were fertile and effective according to morphologic and structural controls (Johnson and Bernard 1962). Consequently, it is very important to point out the relevance of the early molecular characterization by SNP because it allowed us to recognize and to validate the heterozygous F_1 individuals, which supported the accuracy of the results during the complete process of selection and development of the segregating populations, as it was reported specifically in soybean by Yoon et al. (2007). Even more molecular characterization allows inference of the number of genomic regions that are divergent between progenitors and hence, to estimate the possible genetic advance in the corresponding breeding program (Chang et al. 2016).

Finally, results obtained from specific and diverse interactions between *Dpc*16 and soybean genotypes, demonstrated that Ge(13) and Ge(4) were, respectively, the most stable genotypes among the selected R and S parents. Thus, the COD 1-258-2 population, was selected to analyze the *Rdc* inheritance. The chi-square goodness of fit test verified that phenotypic segregation of the complete F_3 population adjusted to a 5:3 ratio (healthy resistant plants: dead susceptible plants) and phenotypic characterization of $F_{2:3}$ families (PT) allowed to infer the genotypic ratio (1RR: 2Rr: 1rr) in the previous F_2 population. The results obtained by classic genetic improvement and molecular assistance contributed to detection and identification of a major resistance gene of simple Mendelian inheritance to SSC-*Dpc,* which was named *Rdc1*. Based on the updated bibliography revision, this is the first report on inheritance of *Rdc* resistance genes to SSC caused by *Dpc*.

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Compliance with the ethical standards The authors declare that ethical standards were followed.

Conflict of interest The authors declare no conflict of interest (financial and non-financial). The rest of ethical statements are not applicable for our research.

Informed consent This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. Neither the manuscript nor the study violates any of the journal policies. All authors have been personally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content.

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