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Identification of SSR markers linked to partial resistance to soybean rust in Brazil from crosses using the resistant genotype IAC 100

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ABSTRACT. Soybean rust is considered a highly aggressive disease in soybean crops. Most research has focused on obtaining resistant genotypes based on dominant or recessive alleles, which provide vertical resistance. The identification of promising crosses that may be used to develop genotypes with horizontal resistance from IAC 100 may help to increase the longevity of the recommended cultivars. However, this type of resistance is limited by environmental variables that may hinder selection. We ranked crosses based on their response to soybean rust using genetic estimates and predicted gains. It was also an objective to identify quantitative trait loci (QTLs) associated with resistance to soybean rust in two generations derived from the same cross. Eighty-seven F_4 progenies from IAC 100 (partial resistance) x BRS Caiapônia (susceptible) cross were field phenotyped. The data divided the DNA samples into two groups for bulked segregant analysis, which was carried out using simple sequence repeat (SSR) primers. A linkage map for the F_4 generation was obtained based on 29 SSR markers, which were distributed into nine linkage groups, covering 285.9 cM of the

genome. Six QTLs were mapped in four of these groups and two of them were responsible for 39% of the phenotypic variance in resistance to soybean rust. The linkage map generated for the F_7 generation was similar to that of the F_4 generation, covering 266 cM. Four of the six QTLs mapped in the F_4 generation were also identified in the F_7 generation, showing that the genomic regions contributing to horizontal resistance to soybean rust are stable.

Key words: *Glycine max*; *Phakopsora pachyrhizi*; Horizontal resistance; Microsatellite markers; Quantitative trait loci; Plant breeding

INTRODUCTION

In global agriculture, there are major challenges in supplying food to an exponentially growing population while minimizing the impact on the environment. Soybean [*Glycine max* (L.) Merrill] is the main oilseed crop produced worldwide and the agricultural species most frequently cultivated in Brazil (CONAB, 2017; USDA, 2017).Several factors limit the production of soybean crops, including pests and diseases. In Brazil, approximately 40 diseases have been identified, which are caused by fungi, bacteria, nematodes and viruses (Henning and Godoy, 2009; Grigolli, 2015). The economic importance of each disease varies from year to year and from region to region depending on the weather conditions. The fungus *Phakopsora pachyrhizi* (Sydow & P. Syd.), which causes soybean rust, is considered the most important pathogen affecting this crop, as it has the potential to reduce yield by more than 75% (Yorinori et al., 2005; Pandey et al., 2010; Hartman et al., 2015; Kimati et al., 2016). Thus, it is a significant threat to national production.

Among the strategies used to control the disease, genetic resistance is considered the most efficient in terms of socioeconomic and environmental aspects. Therefore, soybean genetic improvement programs conducted in Brazil have sought to identify genotypes that provide high yields and show resistance to the disease (Martins and Juliatti, 2012; Morales et al., 2012). Some resistant genotypes have already been developed (Zambolim et al., 1983; Arias et al., 2004; Silva et al., 2007; Morales et al., 2012); however, due to the high genetic variability of the fungus, this resistance is rapidly overcome. Therefore, it is necessary to obtain a larger number of superior genotypes with traits that contribute to high yields even under pathogen exposure. Cultivar IAC 100 has an early cycle, a determined growth type and, according to prior studies (Silva et al., 2007), shows partial resistance to soybean rust. Cultivar BRS Caiapônia, in contrast, has an early cycle and an undetermined growth type and is highly susceptible to rust.

Partial resistance was described by Parlevliet (1979).This type of resistance influences components of resistance that reduce the rate of epidemic development. In Brazil, many studies were conducted under greenhouse and field conditions and demonstrated the partial resistance to soybean rust. This resistance is based on many components (latent period, spores production, severity and AUDPC of the disease evolution (Azevedo et al., 2007; Martins et al., 2007a,b; Silva et al., 2007; Koga et al., 2008). This resistance is different from vertical resistance by *Rpp* genes based on TAN or RB lesions reactions (Hartwig and Bromfield, 1983; Hartwig, 1986). Chang et al. (2016) published the characterization of disease resistance loci in the USDA soybean germplasm collection using

Genome-wide association studies. They studied the public Germplasm Resources Information Network and public SNP data (SoySNP50K). The authors identified SNPs significantly associated with disease ratings from one bacterial disease, five fungal diseases, two diseases caused by nematodes, and three viral diseases. They showed that leucine-rich repeat (LRR) receptor-like kinases and nucleotide-binding site-LRR candidate resistance genes were enriched within the linkage disequilibrium regions of the significant SNPs. They review and present a global view of soybean resistance loci against multiple diseases and discuss the power and the challenges of using GWAS to discover disease resistance and included soybean rust. The same study was conducted on soybeans to white mold resistance and new genes were founded in Brazilian germplasm (Wei et al., 2017).

Little is known about the resistance of soybean to *P. pachyrhizi*, and there are few partial resistance genes reported (Langenbach et al., 2016; Rocha et al., 2016; Vuong et al., 2016). Therefore, basic studies are needed to identify new resistant genotypes as well as the molecular mechanisms involved in the resistance process.

Development of plant breeding strategies has been facilitated by advancements in biotechnology. Before the use of biotechnological tools, plant breeders relied on the phenotypic selection to move desired traits into elite lines. With the advent of restriction enzymes and polymerase chain reaction, DNA markers, such as restriction fragment length polymorphism and simple sequence repeat (SSR), have been used in the construction of genetic linkage maps.

Molecular markers are an important tool in crop improvement programs targeting resistance to soybean rust. These markers, unlike morphological ones, are independent of environmental effects and physiological stage of the plant. They also present high levels of polymorphisms, which are distributed throughout the genome. Microsatellite markers are widely used because they are highly reproducible, easy to use and co-dominant (Alcântara Neto, 2001). Co-dominance is extremely important to distinguish homozygous from heterozygous genotypes (Carneiro and Vieira, 2002; Caixeta et al., 2006). The identification of markers linked to resistance enables assisted selection, decreasing the time required to obtain new cultivars through breeding programs (Arahana et al., 2001; Freire et al., 2008).

Finding and incorporating small-effect genes associated to resistance of soybean to rust disease into new cultivars may extend crop protection. Therefore, we ranked promising soybean crosses based on their response to soybean rust and identified, mapped and quantified the effects of quantitative trait loci (QTLs) involved in resistance to this disease using microsatellite molecular markers.

MATERIAL AND METHODS

Evaluation of genotypes in generations $F_{2:3}$ and $F_{3:4}$ under field conditions from ten crosses by Federer augmented block design (Incomplete blocks)

Genetic material

A total of 565 F_4 progenies, derived from crosses between genotypes BRS Caiapônia, IAC 100, UFUS Impacta, BRS Santa Cruz, BRS Luziânia, M-SOY 9350 and Potenza in ten different combinations (Table 1), were ranked according to their resistance to soybean rust. The crosses were performed in a greenhouse in the year 2007 to obtain the F_1

generations. Generations F_1 and F_2 and parental genotypes were also raised in a greenhouse from 2008 to 2009. The $F_{2:3}$ and $F_{3:4}$ progenies and the parents were evaluated in the crop season 2009/10 under field conditions to study the genetic control of resistance to soybean rust (Martins and Juliatti, 2012). The seeds were collected and re-sown in the year 2011 to generate the F_4 progenies used in this study conducted by Pedigree breeding method.

Table 1. Combinations of the seven soybean genotypes in the ten crosses.

Cross	Combination
1	BRS Caiapônia x IAC 100
2	BRS Caiapônia x UFUS Impacta
3	BRS Santa Cruz x IAC 100
4	BRS Luiziânia x M-SOY 9350
5	BRS Santa Cruz x M-SOY 9350
6	BRS Santa Cruz x Potenza
7	BRS Luiziânia x UFUS Impacta
8	BRS Santa Cruz x UFUS Impacta
9	BRS Caiapônia x Potenza
10	BRS Luiziânia x Potenza

Experimental setup and design

The experiments were conducted at the Mycology and Plant Protection Laboratory (LAMIP) of the Federal University of Uberlândia (UFU), Brazil. The field trials were conducted at the Agroteste experimental station, located at latitude 21º12'58'' S and longitude 45º03'18'' W, 900 m above sea level (F_4 population) and at the Glória Farm (F_7 population), located at 18º56'56" S and 48º12'21" W, 919 m above sea level.

The experiments used an augmented block design with genotypes TMG 801 and TMG 803 as common controls and disease resistance standards. The 565 F_4 progenies, without replicates, and the seven parents, with four replicates each, were distributed into 15 unbalanced blocks. The experimental plot consisted of two rows of 5 m each, with a spacing of 50 cm per genotype. The growing conditions were typical conditions for soybean cultivation (Lopes, 2013). During the experiment, chemical control of pests and manual control of weeds were used, as they became necessary.

To reduce the potential for Septoria disease in vegetative stage, since sowing occurred at a late season, two fungicide applications were performed (Silva et al., 2007). In the first application, a fungicide from the strobilurin (azoxystrobin) chemical group was sprayed with 200 mL of commercial product per hectare, for an application volume of 200 L. In the second application, a mixture of triazole and strobilurin (pyraclostrobin + epoxiconazole) was used at a dose of 500 mL per hectare for an application volume of 200 L. The applications were conducted at V4 and V7 stages (Fehr and Caviness, 1981) using a costal CO_2 pressurized sprayer with TeeJet 11002 nozzles. In stage R3, 20 days after the second fungicide application, all genotypes and populations were inoculated with a urediniospore suspension at a concentration of 10,000 spores per mL. The rust inoculum was obtained from a mixture of pathotypes of the pathogen collected under field conditions at Uberlândia-MG.

Phenotypic evaluation

Disease severity on F_4 and F_7 progenies and on the parents was evaluated according to the percentage of leaf area infected based on the diagrammatic scale proposed by Juliatti (unpublished data), which considers percentages of 0.5, 5, 20, 40, 60 and 80% of infected leaf tissue (Supplementary 1). The severity of the soybean rust was assessed through a visual estimation of three leaves from the middle third of the plant, by three different assessors, totalizing nine leaves per plot, in five plants from two lines with 5 meters. Three assessments were conducted at 60, 80 and 100 days after sowing, corresponding to R4, R5.4 and R6 growth stages, respectively.

Statistical analyses

To determine whether differences between the collected severity data were statistically significant, analysis of variance (ANOVA) was performed. Comparisons were made between treatments, including between parents and progenies for each of the ten crosses and between the ten crosses. To perform ANOVA, the Selegen-REML/BLUP software was used (Resende, 2002). The ANOVA assumptions were tested by using GENES (Cruz, 2006) and SPSS version 8.0 (SPSS Inc., Chicago IL).

The variance components were estimated using the Selegen-REML/BLUP software, which uses the restricted maximum likelihood (REML) method, which is recommended in the case of mixed models with unbalanced data (Verneque, 1994; Duarte et al., 2001). It also uses the optimal procedure for prediction of genetic values (best linear unbiased prediction, BLUP) (Resende, 2002). In the model used, the effect of the block was assumed to be fixed, as the effects of the controls, and the effects of genotypes were assumed to be random. The model was represented with the following formula:

$$\boldsymbol{Y}_{ijk} = \mu + b_j + C_k + g_{i(k)} + e_{ijk} \qquad \text{(Eq. 1)}$$

The variables were defined as follows:

$\boldsymbol{Y}_{ijk}$: the observation generated for the plot of block *j* that received genotype *i* derived from cross *k*;

μ: the constant common to all observations (overall average, under "sum zero" type restrictions for each of the other effects);

b_j: the effect of the *j* block (*j* = 1, 2, ... *b*), assumed to be fixed;

C_k: the effect of cross *k*, including the control (*k*: 1, 2, ..., *c*, *c*+1, *c*+2, ..., *c*+*t*), with *c* being the number of crosses yielding progeny and *t* the number of controls;

$g_{i(k)}$: the effect of genotype (progeny or control) *i*, deriving from cross *k* (*i*: 1, 2, ..., p_k; p_k is the number of genotypes in cross *k*), assumed to be fixed and to be null if *i*is a control, or random with independent distribution $N\left(0, \sigma_{\downarrow}(g_{\downarrow})^{\uparrow 2}\right)$ if *i* is a progeny related to cross *k*; and

e_{ijk}: the experimental random error associated with the *ijk*-th plot, assumed to be independent and identically distributed, under $N^{\mathbb{I}(0,\sigma\mathbb{I}_{\downarrow}e^{\uparrow}2)}$.

This is a mixed model in which the nY_{ijk} observations, represented by a vector,$y_{(nx1)}$, can be described as matrices by using the following generalised linear mixed model (Henderson, 1984):

$$\boldsymbol{y} = \boldsymbol{X}\beta + \boldsymbol{Z}\gamma + \varepsilon \quad \text{(Eq. 2)}$$

with:
$\varepsilon N(\phi, R)$;
$\gamma \sim N(\phi, G)$;
$E(y) = X\beta; eVar(y) = V_{(n)} = ZGZ' + R$.

In this model, all of the fixed effects are included in the parametric vector $\beta_{(px1)}$, and the random effects are in the parametric vector$\gamma_{(qx1)}$, except for the errors that comprise vector $\varepsilon_{(nx1)}$. $X_{(nxp)}$ and $Z_{(nxq)}$ represent the incidence matrices of the effects contained in β and γ, respectively.

The average severity scores at 60, 80 and 100 days after sowing were used to analyze the disease data through the area under the disease progress curve (AUDPC) for the ten crosses, the seven parents and the two controls. The AUDPC was used to describe the epidemics in the progeny of each one of the ten crosses and may be calculated using the following formula (Shaner and Finney, 1977):

$$AUDPC = \sum_{n=1}^{t-1} \left[\left(\frac{Y_i - Y_{i+1}}{2(T_{i+1} - T_i)}\right)\right] \quad \text{(Eq. 3)}$$

The variables are defined as follows:
Y_i: disease proportion at the *i*-th observation;
T_i: time (days) at the *i*-th observation; and
n: total number of observations.

The AUDPC was standardized by dividing the area under the progress curve by the epidemic duration (Tn-T1) (Fry, 1977). The AVACPD software was used to obtain the AUDPC (Torres and Ventura, 1991). The averages were compared using Tukey test with a significance threshold of P < 5% (Tukey, 1953).

Construction of the bulked segregants and genetic mapping of microsatellite markers in the cross BRS Caiapônia x IAC 100

Genetic material

The parents were chosen according to their reaction to soybean rust. The IAC 100 and BRS Caiapônia genotypes showed differences in this trait. Cultivar IAC 100 has an early cycle, a determined growth type and, according to prior studies (Silva et al., 2007), shows partial resistance to soybean rust. Cultivar BRS Caiapônia, in contrast, has an early cycle and an undetermined growth type and is highly susceptible to rust.

A total of 87 $F_{4:5}$ progenies and 70 $F_{7:8}$ progenies were used, derived from crosses between BRS Caiapônia and IAC 100. The crosses were performed in 2007, and further generations were produced in the following years. The $F_{4:5}$ progenies used in this study were seeded in the year 2011, and the $F_{7:8}$ generation was seeded in 2014. Both experiments had a Federer augmented block design (Federer, 1961a,b; Federer and Crossa, 2012).

Inoculum preparation and inoculation with *P. pachyrhizi*

Pathogen inoculation was performed to ensure the incidence of the disease in the field. Because the pathogen of soybean rust is an obligate parasite, the bulk material

containing pathogen isolates was obtained from naturally infected leaves that had abundant sporulation in adjacent fields. After spores were obtained, they were suspended in distilled water and counted in a cell-counting chamber (Neubauer). The suspension was diluted in distilled water to a final concentration of 10^5 spores mL^{-1}. Vegetable oil was used as an adhesive spreader in a 0.5 $mL.L^{-1}$ solution. The sprayer system used was a costal CO_2 pressurized sprayer equipped with TeeJet 11002 nozzles. The soybean rust inoculum was applied between the rows. Inoculation was performed at dusk to avoid making the urediniospores non-viable due to lack of free water on the leaves and low environmental humidity, which is caused by higher temperatures during periods of greater insolation. Spraying occurred when the plants reached the R1 stage (Fehr and Caviness, 1981).

DNA extraction and quantification

Leaf tissue samples from each progeny ($F_{4:5}$ and $F_{7:8}$) and from the parents were collected during the R3 stage, labelled in plastic bags and stored in ultra-freezer (-80ºC) until they were used for nucleic acid extraction. Genomic DNA samples from the parents and progenies were purified according to a protocol based on the cetyltrimethyl ammonium bromide (CTAB) technique proposed by Doyle and Doyle (1990), with a few modifications (Couto et al., 2010). Approximately 50 mg of leaf tissue for each genotype was macerated and placed into 1.5-mL microtubes. To each tube, 650 µL of extraction buffer [Tris 1 M (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl, 2% CTAB ($w.v^{-1}$), 1% PVP ($w.v^{-1}$) and 2% β-mercaptoethanol ($v.v^{-1}$)] were added. The samples in the buffer were incubated at 65ºC for one hour, with agitation every 20 min. Next, we proceeded to the extraction: 650 µL of CIA [chloroform + isoamyl alcohol (24:1 $v.v^{-1}$)] were added, and the tubes were then centrifuged at 10,000 rpm for 10 min. A volume of 600 µL of supernatant of the centrifuged material was transferred to new 1.5-mL microtubes, and the DNA was precipitated with the same volume of cold isopropanol. After precipitation, DNA was centrifuged again at 14,000 rpm for 10 min; the supernatant was discarded, and the DNA was washed with washing solution (ethanol p.a. 76%, 10 mM ammonium acetate) then allowed to rest for 20 min. The material was centrifuged again for 5 min at 10,000 rpm, the supernatant was discarded, and 50 µL of elution buffer (TE) [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)] were added. For better DNA purification, the samples were again precipitated with 5 µL of sodium acetate and 100 µL of ethanol p.a. 95%, then centrifuged at 14,000 rpm for 15 min. The supernatant was discarded, and after the removal of any traces of ethanol, the DNA was resuspended in 50 µL of TE + RNAse (10 $ng.\mu L^{-1}$). The tubes were incubated for 30 min at 37ºC.

After the extraction, the DNA concentration of each sample was determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific), and its integrity was confirmed by 0.8% ($w.v^{-1}$) agarose gel (Invitrogen) electrophoresis. The gels were stained with Gel Red (Biotium). After DNA concentration and quality were determined, the samples were diluted to a final concentration of 25 $ng.\mu L^{-1}$ and stored at -20ºC.

Microsatellite amplification

A total of 93 microsatellite markers, previously identified for the soybean crop, were used in the F_4 population (Supplementary 2). These are distributed in the 20 linkage groups of the consensus soybean genetic map (Cregan et al., 1999; Song et al., 2004). Primer positioning is available at the Soybase database (http://soybase.agron.iastate.edu/). Of these, 38 markers tested in the F_4 generation and associated with partial resistance to soybean rust were used to genotype the plants from the F_7 advanced generation.

The PCR reactions were performed in the MyCycler Thermal Cycler (Bio Rad) using the following conditions: 1X PCR buffer, 1.5 mM magnesium chloride, 0.4 mM dNTPs (40 µM of each nucleotide), 0.3 µM forward primer, 0.3 µM reverse primer, 1 U Taq DNA Polymerase® (Invitrogen), 1 µL of DNA (25 ng) and autoclaved deionised water for a final volume of 12 µL. For fragment amplification, a touchdown (TD) PCR program (Don et al., 1991) was used with annealing temperatures of 45 to 60ºC.

The amplified fragments were separated by 4% ($w.v^{-1}$) agarose gel (Invitrogen) electrophoresis, and the samples were stained with Gel Red (Biotium) diluted 1:500. The generated fragments were visualized in a transilluminator (HoeferMacroVue UV-20), and pictures were taken.

Construction of the bulked segregants

Based on the phenotypic evaluation, 13 resistant and 13 susceptible progenies from the F_4 and from the F_7 generations were selected, and equimolar amounts of DNA were taken from each progeny and combined to form bulked segregants (two bulks for each generation) (Michelmore et al., 1991). Progenies with average severity scores below 20% were considered to be resistant, and those with average scores greater than 60% were considered susceptible (80 days after sowing). The bulks were evaluated together with the parents in the primer tests to identify the polymorphism associated with resistance to *P. pachyrhizi*.

Microsatellite analysis

To determine whether the differences between groups were statistically significant, analysis of variance (ANOVA) was conducted with the Selegen-REML/BLUP software (Resende, 2002). The parents and progenies were included in the analysis.

The markers identified as polymorphic for the parental genotypes and contrasting bulks were used for genotyping the F_4 progeny and the F_7 advanced generation. Marker segregation was evaluated using the model conformity test (χ^2) (Nikulin, 1973) for each marker to determine adequacy of the phenotypic distribution model (1:2:1) (Ramalho et al., 2005). Only markers with adequate segregation were considered for the analyses.

Generation of the genetic linkage map

Once the analyses were completed, a genetic linkage map was generated for the polymorphic microsatellite markers. The map was based on the phenotypic data and was generated using MAPMAKER/Exp software version 3.0 (Lander et al., 1987). The criteria used to form linkage groups were a minimum logarithm of odds (LOD) score of 2.5 and a distance between adjacent marks of 50 cM. The LOD (Lynch and Walsh, 1998) is a significance test that tests the hypothesis of linkage between two loci. It is based on the likelihood ratio and uses a base 10 logarithm. Therefore, a LOD score of 3 indicates that linkage is a thousand times more likely than independent segregation (Carneiro and Vieira, 2002). Studies mapping soybean QTLs usually accept LOD scores between 2.0 and 3.0 to determine a significant association (Zhang et al., 2004; Kassem et al., 2006; Guzman et al., 2007; Gutierrez-Gonzalez et al., 2009).

The Kosambi mapping function (Kosambi, 1944) was used for the conversion of recombination units into genetic distances. The maps obtained for both generations ($F_{4:5}$ and $F_{7:8}$) were compared.

Mapping of QTLs associated with resistance

A search for QTLs contributing to resistance to soybean rust was performed. The WinQTL Cartographer software version 2.5 (Wang et al., 2007) mapping toolset was used primarily for simple marker mapping. To determine the independence of the identified QTLs, composite interval mapping (CIM) was performed. The score was 2.5.

Multiple regression analyses were performed to detect potential interactions and to quantify the effects of the identified QTLs. A probability of 5% was used for the model (Draper and Smith, 1996). To determine the stability of the QTLs in the two generations studied ($F_{4:5}$ and $F_{7:8}$), we compared the magnitudes and locations of the QTLs.

RESULTS

Evaluation of genotypes in generations $F_{2:3}$ and $F_{3:4}$ under field conditions from ten crosses by Federer augmented block design (Incomplete blocks)

Analysis of variance and average components

According to the analysis of variance (ANOVA) performed within each of the crosses (Table 2), there are significant differences in the severity scores between the progenies and their respective parents, except for the cross between the BRS Caiapônia and Potenza genotypes. The main explanation for the non significant values for the F test in this cross is that the parents do not differ in this trait (both are susceptible to rust). Therefore, no significant differences were found between them and their progeny.

Table 2. Summary of the analyses of variance for the severity of soybean rust for the ten different crosses.

No.	Cross	Treatment		Error		Average	CV
		DF	MS	DF	MS	(%)	(%)
1	BRS Caiapônia x IAC 100	2	173.56	68	22.58	36.89**	30.28
2	BRS Caiapônia x UFUS Impacta	2	367.70	57	78.54	48.54*	26.46
3	BRS Santa Cruz x IAC 100	2	736.73	57	156.23	54.38**	24.52
4	BRS Luiziânia x M-SOY 9350	2	363.45	33	111.56	63.85*	34.34
5	BRS Santa Cruz x M-SOY 9350	2	125.76	42	421.60	78.45**	25.76
6	BRS Santa Cruz x Potenza	2	167.81	41	34.63	76.34*	32.64
7	BRS Luiziânia x UFUS Impacta	2	445.77	56	78.46	58.56**	27.33
8	BRS Santa Cruz x UFUS Impacta	2	378.34	58	56.42	86.70**	19.23
9	BRS Caiapônia x Potenza	2	34.63	76	35.66	93.23ns	12.25
10	BRS Luiziânia x Potenza	2	227.58	113	45.77	63.56*	8.34

DF: degrees of freedom; MS: mean square; CV%: coefficient of variation, expressed as a percentage; **, * and ns: significant at 1 and at 5% of probability, and nonsignificant in the F test, respectively.

The crosses BRS Caiapônia x IAC 100 and BRS Caiapônia x UFUS Impacta, which had parents that showed high contrasts in the trait, were the ones that presented better selection gains (SG). The selection of resistant plants originating from these crosses allowed for lower severity averages of the soybean rust. The lowest selection gain, as expected, was estimated for the cross between BRS Caiapônia and Potenza (Table 3). The cross between the BRS Luiziânia and M-SOY 9350 genotypes showed the lowest selection accuracy (SA). This may have been due to the small size of the population that was tested, which also accounted for the overestimation of the heritability.

Table 3. Ranking of crosses and summary of the estimates of the variance components for each of the ten crosses from the genotypes BRS Caiapônia, IAC 100, UFUS Impacta, BRS Santa Cruz, Potenza, M-SOY 9350 and BRS Luiziânia.

No.	Cross	P	GV	SG	SA	σ_g^2	σ_e^2	h^2
1	BRS Caiapônia x IAC 100	1	29.71	-22.17	0.96	5.10	171.73	69.40
2	BRS Caiapônia x UFUS Impacta	2	37.18	-14.70	0.86	1.54	124.78	39.45
3	BRS Santa Cruz x Potenza	5	42.70	-9.17	0.92	10.75	352.57	79.32
4	BRS Santa Cruz x M-SOY 9350	6	42.87	-9.01	0.86	0.29	36.91	34.64
5	BRS Santa Cruz x IAC 100	4	44.08	-7.80	0.95	7.14	189.36	61.57
6	BRS Luiziânia x M-SOY 9350	3	44.10	-7.78	0.74	17.12	82.83	98.11
7	BRS Luiziânia x UFUS Impacta	7	46.25	-5.63	0.95	2.56	226.98	77.78
8	BRS Luiziânia x Potenza	9	47.92	-3.96	0.95	9.57	245.65	89.44
9	BRS Santa Cruz x UFUS Impacta	10	51.21	-0.67	0.91	1.75	46.78	35.40
10	BRS Caiapônia x Potenza	8	51.76	-0.12	0.96	1.46	28.66	30.51
	Average TMG 801	38.25			Average TMG 803			32.33
	Overall average	38.91						
	CV (%)	15.66						

P: position in ranking; GV: genotypic value; SG: estimated selection gain; SA: selection accuracy; σ_g^2: genetic variance; σ_e^2: environmental variance; h^2: heritability, expressed as a percentage; CV: coefficient of variation. Ranking was based on the following genetic parameters: SG, SA and h^2.

Area under the disease progress curve (AUDPC)

The AUDPC is the variable that represents epidemics as a whole. It takes the stress that the crop suffers during different stages of development into consideration

(Bergamin Filho and Amorim, 1996). The AUDPC averages for the crosses, parental genotypes and controls are listed in Table 4.

BRS Luiziânia, M-SOY 9350, Potenza, BRS Caiapônia genotypes and crosses involving these cultivars showed greater susceptibility to soybean rust, whereas IAC 100presented the greatest resistance to the pathogen.

Table 4. Area under the disease progress curve (AUDPC) for the ten crosses, the parents and controls.

Crosses/Genotypes	AUDPC	
BRS Caiapônia x IAC 100	283.94*	ab
BRS Caiapônia x UFUS Impacta	285.67	ab
BRS Santa Cruz x IAC 100	329.50	ab
BRS Luiziânia x M-SOY 9350	713.22	c
BRS Santa Cruz x M-SOY 9350	853.39	d
BRS Santa Cruz x Potenza	751.28	cd
BRS Luiziânia x UFUS Impacta	947.94	d
BRS Santa Cruz x UFUS Impacta	717.28	c
BRS Caiapônia x Potenza	729.17	c
BRS Luiziânia x Potenza	463.78	bc
BRS Caiapônia	627.50	bcd
IAC 100	183.94	a
UFUS Impacta	297.35	ab
BRS Santa Cruz	339.98	bc
BRS Luiziânia	998,06	d
M-SOY 9350	884.44	d
Potenza	856.44	d
TMG 801	218.61	ab
TMG 803	194.35	a

*Averages followed by identical letters do not differ statistically according to Tukey's test at 5% probability.

Construction of the bulked segregants and genetic mapping of microsatellite markers in the cross BRS Caiapôniax IAC 100

Linkage map of the $F_{4:5}$ and $F_{7:8}$ generations

As previously shown, the cross between BRS Caiapônia and IAC 100 showed the best performance. This cross attained the highest predicted selection gain and showed the best SA, and it had high heritability (Table 3). Therefore, we proceed with the genetic mapping of generations derived from this cross.

To identify polymorphisms between the parents IAC 100 and BRS Caiapônia and between the bulk segregant pools, we tested 93 microsatellite molecular markers that were distributed across the 20 soybean linkage groups described by Cregan et al. (1999) and Song et al. (2004). Of these, 52 pairs of primers identified polymorphism between the parents, but only 38 were polymorphic for both the parents and the bulk samples. The other pairs of primers identified polymorphisms linked to other contrasting traits in the parents that are unrelated to resistance to soybean rust. The polymorphic primers were used for genotyping the $F_{4:5}$ progeny and the $F_{7:8}$ advanced generation plants, all of which were previously evaluated in the field for resistance/susceptibility to rust.

Of the 38 markers used, two (Satt440 and Satt260) showed deviations from the expected proportion of individuals (1:2:1) and were not used in the analysis and

construction of the genetic map. Of the remaining 36 markers, seven were not mapped to any linkage group because they had a genetic distance greater than 50 cM (Figures 1 and 2).

Nine linkage groups were detected in the $F_{4:5}$ generation (Figure 1). Marker order and distance may be compared to the integrated soybean linkage map (Song et al., 2004), except for linkage group C2, on which there was an inversion in the order of the Satt277 and Sat_402 markers. The total coverage of the map was 285.9 cM.

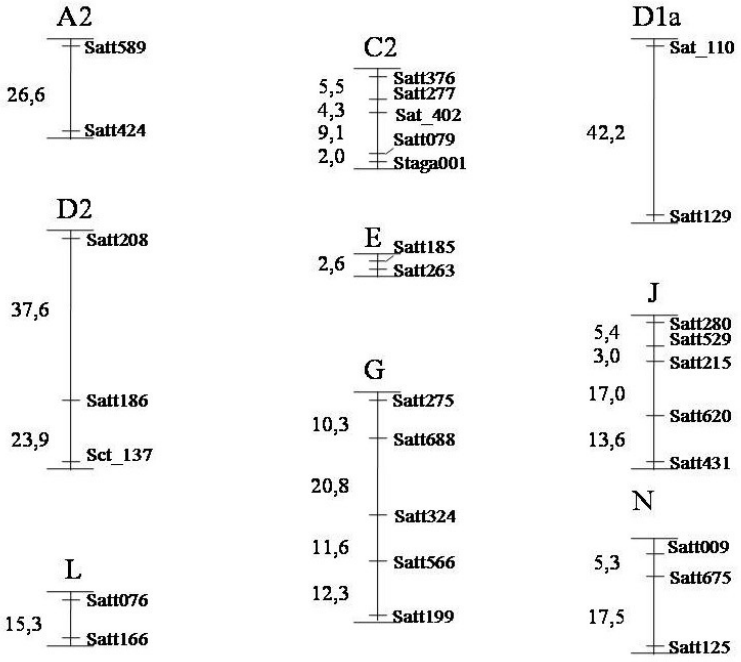

Figure 1. Partial soybean linkage map based on genotyping of 29 SSR markers in an F_4 mapping population originating from the cross between IAC 100 and BRS Caiapônia. The marker loci are shown to the right of each linkage group (A2, C2, D1a, D2, E, G, J, L and N), and the genetic distances (cM) are shown on the left.

The linkage map generated for the $F_{7:8}$ generation was similar to the map for the $F_{4:5}$ generation, covering 266.4 cM of the genome. There were differences in only three of the nine groups: markers Satt199 and Satt566 were inverted in linkage group G, and the distances between markers Sat_110 and Satt129 in linkage group D1a, and between markers Satt208, Satt186 and Sct_137 in the linkage group D2, were lower in the map obtained for the $F_{7:8}$ population. These differences may be due to the low saturation of the map. Four of the six QTLs mapped in the $F_{4:5}$ generation were also identified in the $F_{7:8}$ generation, showing stability for these genomic regions and contributing to the horizontal resistance to soybean rust (Figure 2). Although the genome coverage provided by the 29 SSR markers is low, the data obtained in this study establish the initial stage of tests for QTLs for resistance to soybean rust.

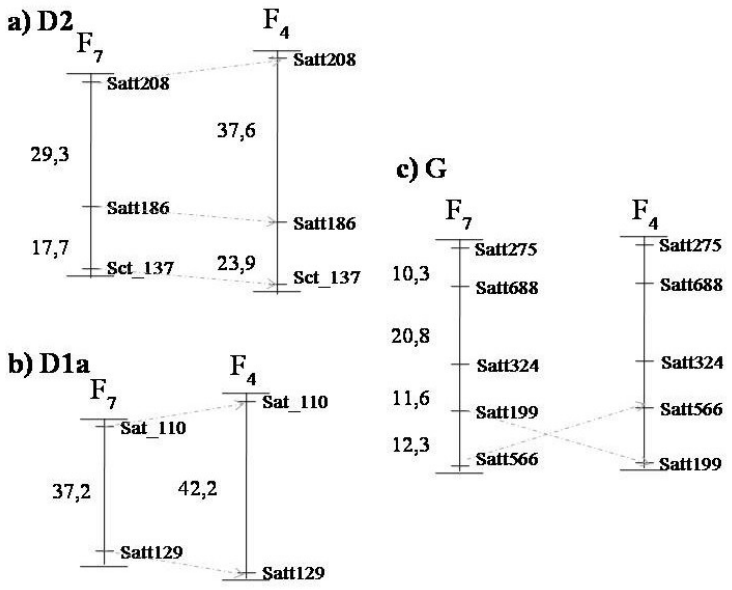

Figure 2 Comparison of linkage groups D2, D1a and G for the $F_{4:5}$ and $F_{7:8}$ populations.

Mapping and comparison of QTLs associated with resistance to soybean rust

In the simple marker QTL mapping, it was possible to identify, in the $F_{4:5}$ generation, 15 peaks in the LODscore curve that exceeded the significance level (2.5) in six linkage groups. In the $F_{7:8}$ population, eight peaks mapped to three linkage groups were identified. Peaks that exceed the limit indicate potential QTLs associated with resistance to the disease. However, in CIM, only six peaks in four different linkage groups (C2, D2, G and N) were confirmed as QTLs in the $F_{4:5}$ generation, and only four (in the groups D2, G and N) were found in the advanced generation. According to multiple regression analysis, the additive effects remain in these QTLs (Table 5 and Figure 3).

Table 5. Description of the significant QTLs detected for resistance to soybean rust in the $F_{4:5}$ (A) and $F_{7:8}$ (B) mapping populations.

A	QTL	LG	Range	Position (cm)	LOD	Additive effect	R^2 (%)
	QTL 1	C2 (6)	Satt376 - Satt277	3.83	4.0	- 0.85	2.80
	QTL 2	C2 (6)	Satt079 - Staga001	19.51	4.3	- 0.58	1.36
	QTL 3	D2 (17)	Satt208 - Satt186	17.20	5.3	- 1.49	10.70
	QTL 4	D2 (17)	Satt186 - Sct_137	48.34	3.6	- 0.27	0.83
	QTL 5	G (18)	Satt688 - Satt324	18.31	10.1	- 6.73	26.35
	QTL 6	N (3)	Satt009 - Satt675	3.00	6.6	- 3.77	12.65
B	**QTL**	**LG**	**Range**	**Position (cm)**	**LOD**	**Additive effect**	**R^2 (%)**
	QTL 1	D2 (17)	Satt208 - Satt186	20.30	7.9	- 4.38	14.50
	QTL 2	D2 (17)	Satt186 - Sct_137	31.20	5.4	- 2.76	5.15
	QTL 3	G (18)	Satt688 - Satt324	20.31	13.1	- 8.73	30.64
	QTL 4	N (3)	Satt675 - Satt125	9.78	7.2	- 4.35	12.77

LG: linkage group, with the values in parentheses corresponding to the chromosome number; LOD score; R^2 (%): coefficient of determination, expressed as a percentage.

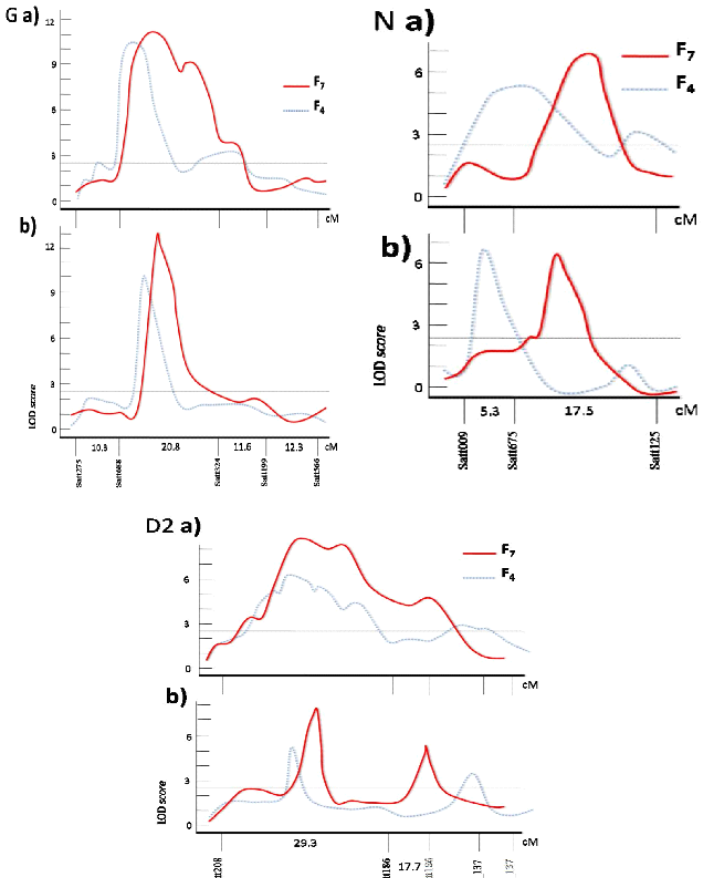

Figure 3. LOD score curves for the severity of soybean rust on linkage groups G, N and D2, in the $F_{4:5}$ and $F_{7:8}$ populations. The distances between the markers, in cM, are indicated between the marks (grey marks represent the distances found for $F_{4:5}$). The LOD (2.5) significance level is indicated by the horizontal dotted line. A) simple marker mapping and B) composite-interval mapping (CIM).

A total of six QTLs for soybean rust resistance were identified and mapped in the $F_{4:5}$ generation in this study. The QTLs explain altogether more than 54% of the variance for the trait, with 39% of the variance explained by two large-effect QTLs (QTL 5 and QTL 6), which are flanked by the markers Satt688, Satt324, Satt009 and Satt675.In the $F_{7:8}$ generation, four QTLs related to partial resistance to soybean rust were identified and mapped. The four QTLs together explain 63.06% of the variance in the trait. The composite-interval analysis showed that two independent QTLs were responsible for the peaks identified in linkage group D2 (chromosome 17). QTL 1, with a larger effect and a LOD score of 7.9, explains 14.5% of the phenotypic variance for severity. In the $F_{4:5}$ generation, a QTL for the same region was identified, but with a smaller effect (10.7%). The second QTL identified in this group explains 5.15% of the phenotypic variance, and it has the effect of decreasing the average severity by 2.76 points.

The simple marker mapping of linkage group G (chromosome 18) showed a large-effect QTL between markers Satt688 and Satt324, and it is responsible for 26.35 and 30.64% of the variation in the trait in the $F_{4:5}$ and $F_{7:8}$ populations, respectively. It has the effect of decreasing the average severity score by 6.73 and 8.73 percentage points in these populations.

DISCUSSION

The analyses showed that the environmental effect overestimates the severity trait, because high coefficients of variation were observed. This may be explained by the high influence of the environment on the trait. High coefficients of variation are frequently obtained for severity assessments (Pierozzi, 2007; Rachid, 2008; Ribeiro, 2009; Melo et al., 2015). Moreover, comparisons between different designs show that the coefficient of variation tends to be higher in the augmented block design (Oliveira, 1993; Bearzoti et al., 1997; Aguiar et al., 2000; Duarte et al., 2001; Peternelli et al., 2009).

Selection accuracy (SA) evaluates the quality of the experiment using a genetic and statistical approach, unlike the coefficient of variation, which uses only a statistical approach. One of the obstacles to obtaining genetic gains in plant improvement is low SA. This parameter corresponds to the correlation between the real genotypic value of the genetic treatment and that which is estimated from the experimental data (Resende, 2004). In our experiment, the SA was high (0.90) for the majority of the crosses, except for BRS Caiapônia x UFUS Impacta (0.86), BRS Santa Cruz x M-SOY 9350 (0.86) and BRS Luiziânia x M-SOY 9350 (0.74). Despite the lowest SA value, heritability observed for the progeny of cross BRS Luiziânia x M-SOY 9350 was high. Duarte et al. (2001) reported that small populations tend to have their variance overestimated, and consequently, the heritability is overestimated. This cross only had 25 $F_{4:5}$ progenies, which may be why the genetic variance and h^2 were overestimated. The same phenomenon may have occurred with crosses BRS Luiziânia x Potenza and BRS Santa Cruz x Potenza.

The genotype that presented the greatest resistance to the pathogen was IAC 100. This cultivar is the result of the Genetic Improvement Program of the Agronomical Institute of Campinas and is considered to be partially resistant to the soybean rust fungus (Silva et al., 2007). Our results confirm this resistance. Cultivar UFUS Impacta, which was developed by the Improvement Program of UFU, shows the second best resistance to the pathogen (aside from the controls).BRS Luiziânia, M-SOY 9350, Potenza, BRS Caiapônia genotypes and crosses involving these cultivars, on the other hand,showed greater susceptibility to soybean rust. The Potenza cultivar was reported to be partially resistant (Silva et al., 2007); however, in this experiment, the rust severity scores for this cultivar were high, suggesting that it is susceptible to rust. The results presented by Martins and Juliatti (2012) also showed that this cultivar is susceptible. The varying results may be related to environmental conditions and/or pathogenic variability (Juliatti et al., 2003; Yorinori and Lazzarotto, 2004).

The TMG 801 and TMG 803 genotypes presented low AUDPC scores, indicating resistance to the trait. This was expected because they were selected as standards of resistance. These genotypes are the result of the Genetic Improvement Programme of the Mato Grosso Foundation and the company TMG (Tropical Melhoramento Genético), and they were developed with Inox technology. These varieties are considered to be resistant to

the soybean rust fungus (Fundação MT, 2009). These results confirm that the technology was a success in these varieties.

In addition to estimating the epidemics through disease severity, AUDPC shows a strong correlation with productivity (Marchi et al., 2008; Godoy et al., 2009; Freitas, 2012). Therefore, in future experiments, we recommend collecting grain yield data for the progeny. It will then be possible to determine the correlation between the severity and productivity data, which would make it easier to identify lineages that are more resistant to the pathogen.

Based on our results, we were able to compare crosses with the best progeny, whose parents may be used in the development of lineages with higher resistance to rust. Crosses between BRS Caiapônia and IAC 100 and between BRS Caiapônia and UFUS Impacta allowed greater selection gains, and these gains were achieved with high accuracy. The cross between IAC 100 and BRS Caiapônia showed the highest heritability for resistance to soybean rust (Tables 3 and 4). Therefore, data from the cross between IAC 100 (partial resistance to *P. pachyrhizi*) and BRS Caiapônia (susceptible to the fungus) were further used for studies focusing on the detection and mapping of QTLs that contribute to the soybean rust horizontal resistance process. The acquisition and use of genotypes resistant to soybean rust may be useful to reduce of the number of fungicide applications (Oliveira et al., 2005; Silva et al., 2007; Martins and Juliatti, 2012; Santos et al., 2018).

Despite the large number of soybean genotypes present in Brazil, studies have reported that the Brazilian germplasm shows low genetic variability. The Brazilian germplasm originates from relatively few ancestors, which means that the population has a narrow genetic base (AlcântaraNeto, 2001; Miranda et al., 2007; Kussler and Bonetti, 2008). These studies corroborate the results obtained in our paper: the low rate of polymorphism between the parents may be partially explained by the narrow genetic base of the soybean crop.

Although our genome coverage was not very wide (285.9 and 266.4 cM), it is possible to find QTLs for traits of interest even in linkage maps with low saturation. Other studies have reported maps with partial coverage of the genome that were used for the mapping of agronomic traits specific for the soybean crop. Brogin (2005) generated a linkage map with 41 SSR markers, linked in nine groups, and mapped QTLs for Septoria brown spot and identified resistance genes for rust. QTLs related to protein and oil content were mapped in nine linkage groups (LG) comprising 25 SSR markers (Rodrigues et al., 2010). Santos et al. (2006) mapped 24 microsatellite markers in six LGs to map QTLs associated with nitrogen fixation. Genes that confer tolerance to water salinity were mapped by Guan et al. (2014) using 12 SSR markers that mapped to LG C. However, the saturation of chromosomal regions that may be related to the QTLs is valid because the effect of each QTL can be better estimated this way.

The differences between the QTLs identified in the two mapping populations ($F_{4:5}$ and $F_{7:8}$) may be associated with the low saturation of the linkage map for both populations, in addition to the instability of the small-effect QTLs. This instability shows a potential interaction between the QTLs and the generations. Moreover, there may be interactions between the QTLs and the environment, because the experiments were conducted at different sites and in different seasons. Austin and Lee (1998) also reported differences in mapping between distinct generations derived from the same cross. These authors detected QTLs of different magnitudes and positions in the $F_{2:3}$ and $F_{6:7}$ generations. In addition, in the $F_{6:7}$ generation, they detected only 13 of the 40 QTLs mapped in the $F_{2:3}$ generation.

In linkage group N ($F_{7:8}$ generation), both the simple marker mapping and CIM showed a significant peak between Satt675 and Satt125, representing a medium-effect QTL that explains 12.77% of the phenotypic variance. This QTL was not mapped in the $F_{4:5}$ generation in this region, but between Satt009 and Satt675. This suggests that there may have been a chromosomal rearrangement between these loci that occurred between generations. However, even with the change in position, the QTL is flanked in the two populations by markers Satt009 and Satt125. The locus represented by marker Satt009 was described as being associated with other QTLs related to resistance to soybean diseases, such as white stem rot (Arahana et al., 2001) and *Phytophthora sojae* (Lee et al., 2013). However, the Satt125 marker, until now, had not been reported as being associated with QTLs involved in resistance to soybean diseases.

The three linkage groups that presented significant QTLs in the $F_{7:8}$ generation (G, D2 and N) also presented significant QTLs in the $F_{4:5}$ generation. Coincidentally, these were the QTLs with the largest effects in the two mapping populations. This result demonstrates the stability of these QTLs.

Takuno et al. (2012) performed simulations of $F_{4:5}$ and $F_{7:8}$ mapping populations and proposed that the identification of QTLs in initial generations is as efficient as in advanced generations, and it may reduce the costs inherent to generational advancement. However, the results obtained in our study show that, even though the large-effect QTLs are coincident in the two mapping generations ($F_{4:5}$ and $F_{7:8}$), small-effect QTLs were identified in the $F_{4:5}$ generation that were not identified in the $F_{7:8}$ generation. Thus, the QTLs are not stable over generations. We suggest that the initial generations should be used carefully for QTL mapping; small-effect QTLs should be discarded to prevent false positives during marker-assisted selection.

Hossain et al. (2015) studied the resistance genes from both PI 594767A and PI 587905. The genes were mapped on chromosome 18 corresponding to the same location as known resistance locus *Rpp1*. Quantitative trait locus (QTL) analysis performed on POP3 identified the putative soybean rust resistance locus in PI 416764 on the defined region of chromosome 6 where *Rpp3* was located. The QTLs detected by the mapping explained about 67-72% of the phenotypic variation in POP3. Cluster analysis based on disease reactions to 64 soybean rust populations demonstrated the presence of at least two types of functional resistant *Rpp1*alleles: strong and weak allele(s), e.g. soybean accession PI 594767A and PI 587905 carry the strong resistant *Rpp1* allele(s). Introducing or pyramiding strong *Rpp1* allele(s) in elite soybean cultivars is expected to be useful against the South American rust population. In this present study, the gene or genes from IAC 100 may be different from the PI genotypes of Hossain and collaborators' studies.

Linkage groups C2 (chromosome 6), G (18), J (16), N (3) and L (19) are known in the literature because they contain vertical genes related to resistance to soybean rust (Garcia et al., 2008; Silva et al., 2008; Hyten et al., 2009; Li et al., 2012; Childs et al., 2018). QTLs were identified in the C2, G and N groups. The QTL closest to one of these resistance genes was QTL 6, which mapped to the N group in a region separated by approximately 20 cM from the region containing the resistance gene *Rpp5* (Garcia et al., 2008; Morceli et al., 2008), according to the soybean consensus map (Song et al., 2004). The other mapped QTLs are found in regions different from those of the *Rpps* genes. The QTLs are chromosomal regions where several small-effect genes are found, and they do not necessarily need to be in the same regions of the genes conferring vertical resistance to the

disease (Garcia et al., 2008). Santos et al. (2018) reported the existence of QTLs in regions different from those mapped for the rust resistance genes. No QTLs were detected for the J nor for the L groups but were found for the D2. The absence of QTLs in these groups may be explained by the lack of saturation of the linkage map because the majority of the markers were concentrated in a single region.

There are few studies focusing on the mapping of QTLs that may influence horizontal resistance to soybean rust. Thus, the data obtained in this study are useful. The markers flanking the chromosomal regions of the large-effect QTLs (Satt208, Satt186, Satt688, Satt324, Satt009, Satt675 and Satt125) may be effective for molecular marker-assisted selection. These QTLs need to be validated in segregating populations at different sitesin order to confirm their effectiveness.

CONCLUSIONS

The best of the ten-genotype combinations originated from the cross between IAC 100 and BRS Caiapônia. This cross showed greater predicted selection gains, high accuracy and good heritability for resistance to soybean rust. Therefore, this combination was chosen for generation advancement to identify polymorphic markers and to map QTLs linked to resistance to soybean rust.

Overall, six QTLs were mapped in the $F_{4:5}$ generation, and four were mapped in the $F_{7:8}$ generation. The four were large-effect QTLs, and they were found in both of the mapping populations. The $F_{7:8}$ generation was more efficient to use for mapping because only the stable and large-effect QTLs were identified.

The most efficient markers for assisted selection were Satt208, Satt186, Satt688 and Satt324 because they selected two stable and large-effect QTLs capable of explaining more than 45% of the phenotypic variance for the trait.

Additional markers should be used to saturate the genetic map. Genome coverage should be increased, and spacing between markers should be decreased. This distance may have hindered the identification of other QTLs present in these regions, and its reduction may refine the location of the identified QTLs. Even though, this study pointed to interesting QTLs that could be further explored to confer resistance to rust disease in soybean genotypes. In the future, GWAS (Genome Wide Associations Studies) analyses could be used to discover disease resistance in soybean to rust and other diseases (Hao-Xun et al., 2016; Wei et al., 2017).

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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