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Inheritance of Resistance of Soybean for *Meloidogyne incognita* and Identification of Molecular Marker for Marker Assisted Selection

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Authors' contributions

This work was carried out in collaboration between all authors. Author LAO made the DNA extraction and PCR analysis and wrote the first draft of the manuscript. Author PV helped in phenotypic analysis and data analysis. Author TDNM performed the phenotypic analysis. Author FL managed the literature searches and helped in PCR analysis. Author IS designed the study, performed the statistical analysis, and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To study the inheritance of resistance to *Meloidogyne incognita* in soybean cultivar CD 201, and identify molecular markers linked to resistance genes/QTLs in soybean.
Study Design: The phenotypic assay was a complete randomized design, and mendelian hypothesis was applied.
Place and Duration of Study: Biotechnology lab, Coodetec, BR 467, km98. Cascavel, PR, Brazil, between July 2012 to July 2013.
Methodology: The population was created by the crossing the cultivars CD 201 (resistant) and BRS 133 (susceptible). $F_{2:3}$ families were phenotyped for resistance to *M. incognita* and

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microsatellite molecular markers were used to identify genes/QTLs associated with resistance. Inheritance hypothesis was tested by Chi square test.
Results: The resistance to *M. incognita* in soybean cultivar CD 201 is given by three epistatic additive genes, two dominant and one recessive. Among the markers, Satt358 is linked to a dominant gene/QTL of resistance explaining 9.9% of the variability in resistance in the evaluated population. The use of this marker allows increasing the frequency resistant or moderately resistant lines in soybean breeding programs. Sixty nine percent of $F_{2:3}$ families that have at least one allele for resistance in marker Satt358 have resistant or moderately resistant phenotype, and no $F_{2:3}$ families that is homozygous with the susceptible allele in this locus have resistant phenotype.
Conclusion: This finding can help soybean breeders to develop highly resistant cultivar to *M. incognita*, both, by phenotypic selection and marker assisted selection.

Keywords: Glicyne max; root knot nematode; genetic hypothesis; molecular breeding; marker assisted selection.

1. INTRODUCTION

Brazil is the second largest producer and exporter of soybeans in the world [1]. The high levels of productivity in the country are due to investments in technologies that fight the factors that limit the production, the development of resistant plants to several types of biotic stresses, including galls nematodes (*Meloidogyne spp*), which accounted for economic losses above $ 52 million in 1999/2000 harvest [2].

Meloidogyne incognita (Kofoid & White) Chitwood is considered one of *Meloidogyne* species of greatest economic importance in the world. This pathogen is commonly found in tropical and temperate regions [3]. Four different pathogen races were identified [4], which are reproduced primarily in tobacco and cotton [5].

The study and the identification of native genes to genetic resistance has been the primary means employed to control the pathogen in different cultures. The knowledge of inheritance of the resistance and the identification and validation of molecular markers, which can be applied in various breeding programs, is an important tool for obtaining genetic resistance, and it is essential in the management of crops to obtain high productivity and yield stability in various environments [6].

Genetic resistance to nematodes is considered one of the most efficient and cost-effective methods to prevent production losses [7]. The evaluation of resistance to nematodes is a complex activity, costly and time-consuming as well as being heavily influenced by the environment. For this reason, it is difficult to use phenotypic selection in large-scale soybean breeding programs. Most soybean genotypes from Brazil has a history of susceptibility to *Meloidogyne javanica* and *M. incognita* [8], despite the availability of resistant germplasm. However, the difficulty to select resistant breeding lines has hindered the obtaining varieties with high levels of resistance to producers [9].

The identification and validation of molecular markers associated with genes or QTLs for resistance can make an important contribution to soybean breeding programs to increase the frequency of resistant lines in their progenies [10]. The markers assisted selection is simpler, faster, and more economic than the phenotypic selection, and is not influenced by the environment variation. Another important factor is that the distinction between resistance and susceptibility is not always easily obtained, particularly at moderate levels of resistance or susceptibility in phenotypic evaluation [7].

Studies of heritability of resistance to nematode galls in soybean were performed for different sources of resistance [11,12,13,14]. Among these studies, it was identified the location of genes in soybean linkage groups and consequently the potential molecular markers were tested for association to soybean resistance to these nematodes [13,15]. These markers need to be tested and validated for each new source of resistance before applying in the breeding program [16]. Thus, each program must study and validate the sources of resistance to the nematode galls, to check which genes and molecular markers are effectively linked and allow selection of resistant plants.

This work aimed to study the inheritance of resistance to *M. incognita* in cultivar CD 201, and

identify/validate molecular markers linked to genes/QTLs for resistance to be used in marker assisted selection in soybean breeding programs.

2. MATERIALS AND METHODS

The experiments were carried out in a greenhouse and laboratories of Coodetec, in Cascavel, Paraná state, in Brazil (24º52'55, 44''S; 53º32'21, 44'' W).

2.1 Population Development

The studied population was obtained by crossing varieties CD 201 (resistant to *M. incognita* race 3) and BRS 133 (susceptible). The F_1 and F_2 were grown in a greenhouse. In F_2, 151 plants were selfed, and the seeds obtained in each F_3 plants constituted the $F_{2:3}$ families, used in genetic studies. Confirmation of the cross was checked by molecular and phenotypic markers in the F_1 plants.

2.2 Phenotyping of the Studied Population

The reaction of plants of the $F_{2:3}$ families to *M. incognita*, was conducted in a greenhouse at a 26ºC. Plastic tubes (25 cm length, 5 cm diameter) were used with substrates containing building sand and tillage soil in the ratio of 2:1 (v/v), and one plant per tube. Seven plants of each family were sown and after 10 days were inoculated with 5000 nematode eggs per plant. The phenotypic evaluations were performed 40 days after the inoculation. The plants were removed from the tubes, the excess of sand and soil was carefully removed and the roots were washed and separated by family.

To determine infestation of nematode galls in the roots of $F_{2:3}$ families, the scoring system proposed by USDA [17] where used: 1 = <10% of the root system, with small galls; 2 = 10-25% of the root system galled, and most being small galls; 3 = 26-50% of the root system with large galls; 4 = 51-90% of the root system with large galls; 5 = 91-100% of root system with large galls and necrotic roots.

The class of resistance/susceptibility of $F_{2:3}$ families to nematode galls was determined based on the average score of gall for each family: Resistant (R), score 1 to 1.9; moderately resistant (MR), score 2.0 to 2.9; Moderately Susceptible (MS), score 3.0 to 3.9; Susceptible (S), score 4.0 to 5.0.

2.3 Genotyping the Population with Molecular Markers

Young leaves of plant of the $F_{2:3}$ families were collected in bulk, in every family. DNA was obtained using the protocol proposed by Doyle and Doyle [18]. The Polymerase Chain Reaction (PCR) was conducted in Thermo Hybaid thermocycler (Ashford, Middlesex, UK) and amplified fragments were genotyped on agarose gel or acrylamide gel, according to fragment size.

To assess polymorphism between the parental varieties, 368 microsatellite markers were used. Polymorphic markers between soybean cultivars CD 201 and BRS 133 were used to amplify the DNA of a set of 46 $F_{2:3}$ families, composed by 23 extreme resistant families and 23 extremes susceptible families. Markers showing some association with resistance to galls nematode in this group of extreme individuals were amplified in the entire population. Because of the quality of the amplification, only 129 $F_{2:3}$ families was considered for genotyping.

2.4 Statistical Analysis

The normality of galls scores was tested by Lilliefors test. Segregation analysis was performed using the chi-square test. The combination of molecular markers and nematode galls resistance was evaluated by analysis of simple marks, using linear regression. The analysis was performed with the software's Genes [19] and GQ Mol [20].

3. RESULTS AND DISCUSSION

The frequency distribution of galls scores in the 159 $F_{2:3}$ families (Fig. 1) showed normal distribution by Lilliefors test (P> 20%). Soybean resistance to nematode galls is a trait with quantitative phenotypic expression. However, in the selection for resistance to nematode galls the plants are rated on a qualitative scale, according to the scores of galls.

In the practice of the breeding programs, a qualitative approach is used to evaluate the resistance to nematode galls in soybean, segregating the plants in resistant or susceptible groups. For this reason a qualitative genetic approach was undertaken to assess the segregating population of $F_{2:3}$ families in these study. Firstly, we consider two classes, resistant (R) and susceptible (S). The last class contains the families MR, MS, S, i.e., all classes different from R. In phenotypic analysis were obtained 24

families R and 135 families in the sum of the other classes, which is compatible with the segregation of two genes, one dominant gene and one recessive gene (expected ratio of 3R:13S, χ^2 = 1.39 P = 23.76%). However, with this approach it is not possible to set a hypothesis to explain the differences between the classes MR, MS and S, in the genetic base.

To try to explain the differences between MR, MS and S classes, it was tested the hypothesis of three genes. In Table 1 are presented the hypothesis test for segregation of three genes. This hypothesis explains the variation in the phenotypic classes in terms of number of resistance genes (P = 91.73%). Resistance (R) requires three genes (two dominant and one recessive). These genes are additive and epistatic, that means all three genes are needed for complete resistant. For the MR class, it needs two genes and for the MS class one gene is sufficient. The S class has no resistance gene. In this case, the resistance gene (dominant or recessive) had similar effect on the phenotype and is additive, so that for the MR class, which requires two genes, there is no difference between dominant and recessive genes. This is a hypothesis of oligogenic resistance type, in which three genes are required for full manifestation of the resistance trait, as in resistance to soybean cyst nematode on some cultivars of soybean. The differences in phenotypic expression of the resistance gene may also be due to minor or major effect of the gene [6].

With these results, we conclude that resistance to nematode *M. incognita* present in the variety CD 201 is given for three additive genes, two dominant and one recessive. To complete resistance (R) plants need the three genes. Two genes confer moderate resistance (MR), and plants with one gene are moderately susceptible (MS). Susceptible plants (S) have no resistance gene.

Forty-eight out of 368 microsatellite markers used to evaluate the resistant and susceptible parents (CD 201 and BRS 133) were polymorphic between the parents and were used for genotyping the $F_{2:3}$ families that showed the extreme phenotypes for resistance and susceptibility to *M. incognita*. The marker Satt358 (linkage group O, chromosome 10) were significantly associated with de phenotype on linear regression analysis of the extremes of the population. This marker was used for genotyping 129 $F_{2:3}$ families. In the evaluation of these population, the marker Satt358 was associated with the resistance (P = 0.027; R^2 = 9.9%). This marker explains 9.9% of the phenotypic variation of soybean resistance to nematode *M. incognita*.

Fig. 2 depicts the frequency distribution of phenotypic classes of resistance to *M. incognita* in each group of $F_{2:3}$ families grouped by the marker Satt358 genotype. Of the 20 families with phenotype R, 16 have homozygous genotype equal to the resistant parent genotype (CD201), and the other four are heterozygous for marker Satt358. No resistant plant was homozygous genotype equal to the susceptible parent (BRS133). This result is consistent with the need for a dominant gene for the plants to be resistant. Plants that do not have this dominant gene are susceptible.

Table 1. Hypothesis test for the inheritance of resistance of soybean (cultivar CD 201) to nematode galls *M. incognita*. The hypothesis considers three genes for resistance, two dominant and one recessive (A_B_cc). The number of resistance genes is related with the phenotypic class

Class	Observed	Genotype*	Expected frequency	Expected number	Number of resistance genes	χ^2	P
R	24	***A_ B_ cc***	9	22.3593	3		
MR	78	***A_ B_*** C_	27	81.9843	2		
		A_ bb ***cc***	3				
		aa ***B_ cc***	3				
MS	50	***A_*** bb C_	9	47.2031	1	0.5072	91.7287%
		aa ***B_*** C_	9				
		aa bb ***cc***	1				
S	7	aa bb C_	3	7.4531	0		
Total	159		64	129			

**In italic bold, the resistance alleles, R = resistant; MR = moderately resistant; MS = moderately susceptible; S = susceptible*

The results are also consistent with the need of two genes for MR class. Of the 61 MR plants, 82% have at least one allele for resistance from marker Satt358. The remaining 18% of plants having MR phenotype do not have the dominant allele of resistance on linkage group O. These plants may have the other two genes necessary. Plants carrying the resistance allele at the marker locus Satt358, just need to have one more gene/QTL to be MR, which is more likely to occur than the presence of the other two genes in plants lacking the gene/QTL linked to marker Satt358.

Plants with phenotype MS are proportionally distributed in the three groups of genotypes for Satt358 marker. This result is also consistent with the hypothesis that requires one resistance gene for MS phenotype. The plants which have the gene/QTL linked to marker Satt358 can have no another gene for resistance to have phenotype MS. The plants that lack the gene/QTL linked to marker Satt358 must have one of the other two resistance genes. These probabilities are equivalent.

Four families have the homozygous susceptible alleles, and have R or MR phenotype. It was not expected, and will be explained by recombination between marker and gene/QTL.

The cultivars resistant to nematode galls cropped in Brazil, basically descended from the same source of resistance, the American cultivar Bragg [21,22]. So, it has a narrow genetic base. This suggests that resistance to nematode galls can be controlled by a small number of major genes, even though the phenotypic expression is quantitative. The same occurs with resistance to cyst nematode (*Heterodera glycines*). The *rpp1* and *Rpp4* genes were identified as main genes for soybean resistance to soybean cyst nematode (SCN), but in many cases, a single gene is sufficient for resistance, although the phenotypic expression is quantitative [23]. Phenotypic expression is influenced by the environment (quantity and viability of the inoculum that reaches the roots, root development, temperature, moisture, etc.) and not only the presence of resistance genes in plants.

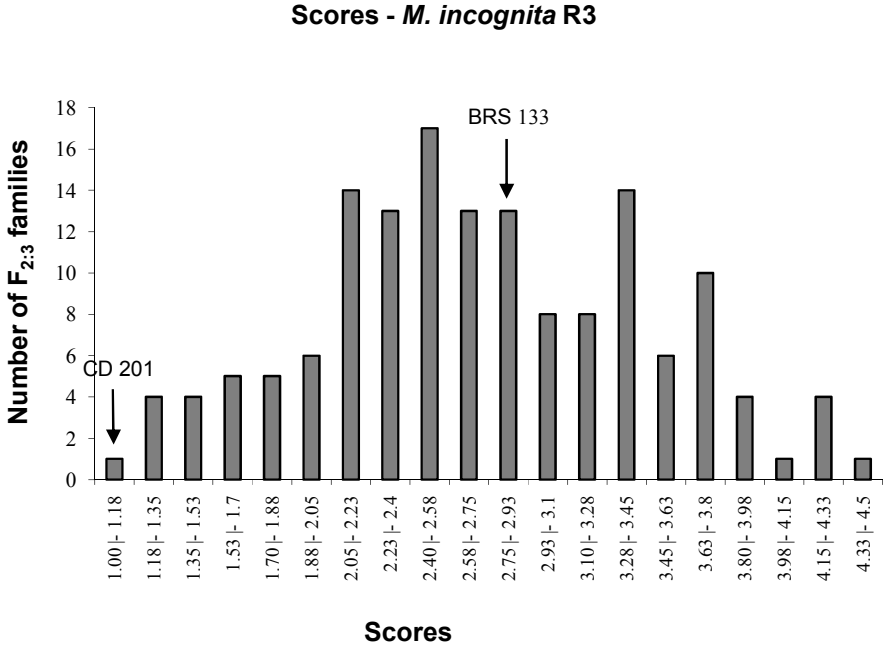

Fig. 1. Distribution of frequency for scores of gall in soybean roots of 151 $F_{2:3}$ families derived from the cross between the cultivar CD 210 (resistant) and BRS 133 (susceptible), evaluated or resistance to *M. incognita* Race 3. The arrow indicates the scores of the cultivars CD 201 and BRS 133

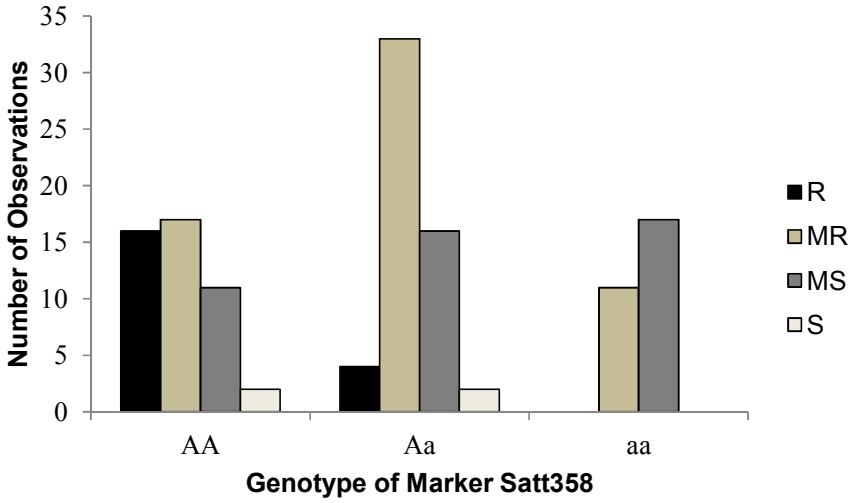

Fig. 2. Frequency of phenotypic class for resistance of soybean to nematode *M. incognita* in each genotype of marker Satt358. The genotype AA represents the homozygous like the resistant cultivar CD 201, and the genotype aa represents the homozygous like the susceptible cultivar BRS 133

The nematode galls *M. incognita* is economically important also in soybean production fields in the United States [24], encouraging several studies on genetic resistance. These studies indicated that resistance to these nematodes have high heritability, with few genes involved [11,12,13, 14]. Luzzi et al. [11], found that the moderate resistance of cultivar Forest is determined by a single gene (*Rmi1*/*rmi1*), located in the linkage group O, with the dominant allele being responsible for the moderate resistance. Li et al. [13] and Ha et al. [15] identified loci for resistance to nematode galls on linkage groups O and G. The QTL in the linkage group O is located near the Satt358 marker, and the QTL in linkage group G is close to Satt199 and Satt012 markers. The markers from linkage group G were monomorphic among the parents of the population used in this study, and the marker Satt358 confirmed the association with resistance to nematode galls. Thus, cultivar CD 201 must possess the gene *Rmi1* in linkage group O.

The use of marker assisted selection in breeding programs requires that the markers are validated on the local germplasm. With the results obtained in this work, one can validate the use of marker Satt358 in selecting plants for resistance to nematode *M. incognita*. This marker has been reported to be linked to resistance to this nematode on North American germplasm, and in this work this linkage was demonstrated in tropical germplasm. The Brazilian soybean germplasm resistant to galls nematode has narrow genetic base, and this marker can potentially be used throughout the Brazilian soybean germplasm for selection to *M. incognita* resistance. Other markers are needed to select the other loci of resistance, but it is possible to increase the frequency of resistant lines with the selection through Satt358 marker. Pham et al. [24] in a study of genetic mapping and identification of genes that control resistance to *M. incognita* successfully used the Satt358 and Satt492 markers to identify recombinant events in a RIL population, obtained by the crossing of PI96354 resistant cultivar with Bossier, a highly susceptible cultivar.

Since it is necessary three genes/QTLs for complete resistance, there is a need to identify markers associated with other genes/QTLs, in order to select resistant plants by molecular markers. But the use of marker Satt358 alone increases the probability of obtaining resistant lines. Between the plants that have the resistance allele at locus Satt358 (homozygous or heterozygous), 20% are R, and 49% are MR, adding 69% R or MR. Among the plants that do not have the resistance allele in the locus Satt358, none presented R phenotype, and only 11% had MR phenotype.

The marker assisted selection for resistance to nematodes can accelerate the selection in the breeding programs, can be performed in the early stages of breeding programs, and does not require maintenance of populations of nematodes and multiplication and inoculation of the plants to know the phenotype [25]. With this method, a larger number of plants can be evaluated by molecular marker, especially in the early stages, increasing the efficiency of soybean breeding programs.

Once the selection assisted by the marker Satt358 has no absolute efficiency, the lines selected by molecular markers need to be confirmed by phenotypic analysis. But it can greatly reduce the amount of lines to be phenotypically evaluated, because only those with the resistance allele for the marker Satt358 need to be evaluated by phenotype. These lines have a high probability to be resistant. The lines that are homozygous for the allele associated with susceptibility in the marker Satt358 do not need to be evaluated phenotypically, because these lines have a very low probability to be resistant and a low probability to be moderately resistant.

4. CONCLUSION

The resistance of the soybean cultivar CD 201 to nematode galls *M. incognita* is given by three genes, two dominant and one recessive. The number of resistance genes explain the difference between the resistant, moderately resistant, moderately susceptible and susceptible phenotype.

One of these genes is *Rmi1*, mapped in linkage group O, near the marker Satt358.

The marker Satt358 can be used in molecular assisted selection to increase the frequency of resistant lines in breeding programs. It can reduce significantly the need of phenotypic evaluation.

These results can be used in Marker Assisted Selection to obtain soybean cultivars resistant to *M. incognita*. It could reduce the loss of productivity for the farmers, caused by *M. incognita*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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