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Genetic variability and resistance of cultivars of cowpea [*Vigna unguiculata* (L.) Walp] to cowpea weevil (*Callosobruchus maculatus* Fabr.)

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ABSTRACT. The cowpea weevil (*Callosobruchus maculatus* Fabr.) is the most destructive pest of the cowpea bean; it reduces seed quality. To control this pest, resistance testing combined with genetic analysis using molecular markers has been widely applied in research. Among the markers that show reliable results, the inter-simple sequence repeats (ISSRs) (microsatellites) are noteworthy. This study was performed to evaluate the resistance of 27 cultivars of cowpea bean to cowpea weevil. We tested the resistance related to the genetic variability of these cultivars using ISSR markers. To analyze the resistance of cultivars to weevil, a completely randomized test design with 4 replicates and 27 treatments was adopted. Five pairs of the insect were placed in 30 grains per replicate. Analysis of variance showed that the number of eggs and emerged insects were significantly different in the treatments, and the

means were compared by statistical tests. The analysis of the large genetic variability in all cultivars resulted in the formation of different groups. The test of resistance showed that the cultivar Inhuma was the most sensitive to both number of eggs and number of emerged adults, while the TE96-290-12-G and MNC99-537-F4 (BRS Tumucumaque) cultivars were the least sensitive to the number of eggs and the number of emerged insects, respectively.

Key words: Inter simple sequence repeat; *Vigna unguiculata*; *Callosobruchus maculates*

INTRODUCTION

Brazil is the second largest producer of cowpea (*Vigna unguiculata*), second only to India. It is a staple food for the rural and urban populations in the northeast of Brazil, is considered the most important grain legume, and supplies part of the protein needs of the poorest populations in this region (Oliveira et al., 1984; Araújo and Watt, 1988; Oliveira and Carvalho, 1988; Quin, 1997).

In order to ensure good productivity, it is very important to choose the correct plant variety for a particular environment and production system. Reduced production costs of culture can be optimized by using better technology, such as using upright plant varieties, which facilitate mechanized harvesting, combined with high resistance to pests, such as those that attack the grain (Santos and Vieira, 1971; Santos et al., 1978). It is also desirable that the cultivar in use provides resistance to diseases that are caused by various agents while meeting market and consumer demands (Freire Filho et al., 2000).

Cowpea is significantly affected by viruses, nematodes, fungi, and bacteria; however, one of the most destructive pests, undoubtedly, is the cowpea weevil (*Callosobruchus maculatus* Fabr.), which reduces the weight and the germinative power of the seed (Dongre et al., 1996). Cowpea weevil infestation, which in some cases starts in pods while still in the field, reduces the weight of the seeds, thereby causing a decline of the nutritional value of the grains (Araújo et al., 1984; Barreto and Quinderé, 2000; Sousa et al., 2008).

In order to increase productivity, there is a need for cultivars with resistance to biotic and abiotic factors, and genetic analysis is an important tool to detect this variability and aspects related to the architecture of the plant (Pessoa et al., 1993; Sanon et al., 2002). An important application of genetics has been molecular markers; inter simple sequence repeat (ISSR) markers stand out because they provide versatile, fast, and reliable results and are effective in diagnosis (Zietkiewicz et al., 1994; Bornet and Branchard, 2001; Reddy et al., 2002; Wolfe et al., 2005; Caixeta et al., 2006). Because the procedure is simple to implement-amplifying specific regions of the genome using small amounts of DNA and samples consisting of several individuals, this technique is advantageous in relation to the cost and time needed to obtain results (Ramos et al., 2006).

Because the susceptibility of the crop to pests, mainly cowpea weevil, is high, little is known about tolerant cultivars, and the most used means of control usually involve high costs for the producer and often include chemical pesticides. It is greatly important to develop resistant cultivars through the discovery of associated molecular markers (Barreto and Quinderé,

2000). This study aimed to evaluate the resistance of cowpea to the cowpea weevil and relates this to the genetic diversity of different cultivars using ISSR markers.

MATERIAL AND METHODS

The research was carried out in the Genomics and Entomology laboratories of the Instituto Agronômico de Pernambuco (IPA). The cultivars studied, sent by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) Região Norte, were grown in the municipalities of Petrolina, Salgueiro, and Araripina, in the State of Pernambuco.

Analysis of cowpea resistance to cowpea weevil

To analyze the resistance of cowpea to the cowpea weevil, we adopted a fully randomized experimental design with 4 replicates (A, B, C, and D) for each cultivar and a control group. Samples of 30 grains for each accession of cowpea were placed in 240-mL glass containers, covered with fabric, and kept at room temperature. Five pairs of 1-day-old cowpea weevils (24 h) were placed in each container. After 72 h, the insects were removed, and 6 days later, an egg count was performed. The experiment was observed daily until the 23rd day, and from the moment that the cowpea weevils began to emerge, they were counted and removed from the sample. Samples from the control group were not infested with cowpea weevils, but they were kept under the same experimental conditions.

Statistical analysis of the resistance test

With the help of software for statistical analysis, the data that were obtained for the variables of the number of eggs and the number of emerged insects were transformed to log (x+1), the variance was analyzed by the F-test, and the means were compared by the Tukey and Duncan tests at the 5% probability level. The results for comparative and statistical analyses of cultivars were organized into tables that were elaborated by the program.

Nuclear DNA extraction

Nuclear DNA extraction was performed following the protocol that was proposed by Ferreira and Grattapaglia (1998): for each sample, 700 µL 2X cetyltrimethylammonium bromide (2X CTAB) extraction buffer was added, samples were heated to 60°C, and 2-mercaptoethanol was added before use to the required amount of buffer at a proportion of 2 µL/mL.

The extraction material, around 150 mg foliar tissue from each cultivar, was ground to a fine powder using liquid nitrogen, a crucible, and a pestle. After maceration, samples were collected in Eppendorf tubes containing the 2X CTAB extraction buffer plus 2-mercaptoethanol. The Eppendorf tubes were incubated in a water bath at 60°C for 30 min and shaken every 10 min to homogenize the suspension.

In a fume hood, the first extraction was made with 600 µL 24:1 chloroform:isoamyl alcohol (CIA). Then, the Eppendorf tubes were shaken for 5 min to mix the emulsion and centrifuged at a maximum speed of 14,000 rpm for 5 min. After being removed from the centrifuge tubes, the upper (aqueous) phase of each sample was transferred to a new Eppendorf tube. Ex-

traction was repeated again with 600 µL CIA, shaking the samples for 5 min, and centrifuging for 5 min. Subsequently, the aqueous phase of each tube was transferred to a new Eppendorf tube.

Approximately 400 µL cold isopropanol, which was equivalent to 2/3 the volume of the aqueous solution, was added to each Eppendorf tube. After the addition of isopropanol, the tubes were centrifuged at 7000 rpm for 5 min to form a pellet. Following this, the supernatant was discarded, and the pellet washed twice in 1 mL 70% ethanol for 5 min and again in 1 mL absolute ethanol for 3 min. After the ethanol was discarded, the pellet was dried in a fume hood for 30 min and then resuspended in 100 µL Tris and ethylenediaminetetraacetic acid (EDTA) + RNAse. The tubes were incubated in a water bath for DNA digestion for 120 min at 37°C; then, they were removed and stored at -20°C.

Nuclear DNA amplification

The DNA samples were amplified by polymerase chain reaction (PCR) using the primers described in Table 1 and following the protocol described in Table 2. Amplification for GTG5 was performed using the following protocol: a denaturation cycle at 94°C for 5 min; 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 45 s, and extension at 72°C for 90 s; and a final extension at 72°C for 6 min. Amplification with the GACA4 and M13 primers was the same except that the annealing temperature was 45°C. For all other primers, the standard programming for ISSR markers was used: denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 2 min; and a final extension at 72°C for 7 min.

Table 1. Primers used for DNA amplification of cowpea cultivars.

Primer	Sequence	Molecular marker	Reference
01	ACACACACACA ACACT	ISSR	Universal
842	GAGAGAGAGAGAGAG	ISSR	Universal
860	TGTGTGTGTGTGTGT	ISSR	Universal
862	AGCAGCAGCAGCAGC	ISSR	Universal
868	GAAGAAGAAGAAGAA	ISSR	Universal
878	GGATGGATGGATGGA	ISSR	Universal
878	GGATGGATGGATGGA	ISSR	Universal
886	CTCCTCCTCCTCCTC	ISSR	Universal
GTG5	GTGGTGGTGGTGGTG	ISSR	Lieckfeldt et al. (1993)
CGACA4	GACAGACAGACAGACA	ISSR	Meyer and Mitchell (1995)
M13	GTAAAACGACGGCCA	ISSR	Lieckfeldt et al. (1993)

ISSR = Inter-simple sequence repeat.

The ISSR amplification was performed with the primers listed in Table 1 and a 24-µL final volume for each sample in a HIBAID thermal cycler according to the protocol described in Table 2. The amplification products were stained with SYBR and separated by electrophoresis on a 1.3% agarose gel in 0.5X Tris, borate, EDTA buffer and subjected to 75 volts/cm for 150 min. After being withdrawn from the box, the gel was set in an ultraviolet transilluminator and photographed.

Statistical analysis of the amplification products

The amplifications with the primers 01, 842, 860, 862, 868, 878, 886, GTG5, GACA4,

and M13 were analyzed with the numerical taxonomy system of multivariate programs-NTSYS-PC 2.1 (Bussab et al., 1990; Rohlf, 1998). The data were transformed into binary variables, in which the number 1 (one) indicates the presence of the band and the number 0 (zero) indicates the absence of the band. A similarity matrix was built using the Jacard similarity coefficient (J), which was calculated according to the formula: J = A / P - D, where *A* indicates the variables that were present, *P* indicates the number of variables, and *D* indicates the variables that were absent. From the similarity matrix, a dendrogram was generated by the unweighted pair group method with arithmetic average clustering method.

RESULTS AND DISCUSSION

Twenty-seven cowpea cultivars were tested for resistance to cowpea weevil, and the sensitivity level was estimated through the variables of the number of eggs and the number of emerging insects. In Tables 2 and 3 presented below, the variables are compared by the Tukey and Duncan tests at 5% probability.

In Table 1, according to the average number of eggs for each cultivar, it is possible to separate the data into 3 distinct groups: A, AB, and B. Group A is composed only of the Inhuma cultivar, which showed the highest number of eggs in relation to the others, while group AB is home to the largest number of cultivars, where there is no significant difference among the means. Group B, however, is composed of the EVX-63-10E, MNC99-510G-8, Patativa, MNC99-537-F4, and TE96-290-12-G cultivars, which exhibited the fewest eggs.

According to Lima et al. (2001), the number of viable eggs in beans can indicate a greater possibility that the plant cultivar will be susceptible to the pest; therefore, there is a

Table 2. Statistical analysis of the egg number variable (Tukey test at 5% probability level).

Treatments	Repetitions	Means	Standard error	Tukey (5%)	Duncan (5%)
07-Inhuma	4	227.25	33.11	a	a
27-TE97-303G-12-BRS-Pageú	4	153.75	48.40	ab	ab
24-Maravilha	4	152.50	20.67	ab	ab
20-Sempre Verde L. E. Magalhães	4	150.25	16.38	ab	ab
01-IPA 207	4	137.50	17.16	ab	ab
08-Pingo-de-Ouro 2	4	134.50	37.74	ab	abc
25-MNC99-508G-1	4	126.50	71.00	ab	abcd
21-Rajado	4	117.25	45.03	ab	abcd
15-MNC99-519D-1-1-5	4	110.75	20.63	ab	abcd
22-Sopinha	4	109.50	26.05	ab	abcd
09-Pingo-de-Ouro 1-2	4	107.25	46.45	ab	abcd
03-BRS-Marataoã	4	104.75	23.52	ab	bcd
04-Paulistinha	4	100.50	39.25	ab	bcd
26-TE97-304G-4	4	99.00	38.91	ab	bcd
23-Vigna 384	4	98.75	26.03	ab	bcd
11-EVX91-2E-2	4	92.50	19.17	ab	bcd
13-MNC00-553D-8-1-2-3	4	84.75	22.97	ab	bcde
19-Pretinho	4	83.75	12.96	ab	bcde
16-BRS-Novaera	4	82.75	13.93	ab	bcde
14-BRS-Potengí	4	79.00	13.04	ab	bcde
02-BRS Milênio	4	66.75	15.23	ab	bcde
05-BRS-Paraguaçu	4	55.00	13.37	ab	bcde
10-EVX-63-10E	4	44.25	14.92	b	cde
17-MNC99-510G-8	4	43.25	21.66	b	cde
06-Patativa	4	39.25	9.94	b	de
12-MNC99-537-F4	4	30.50	17.84	b	e
18-TE96-290-12G	4	26.00	17.35	b	e

greater likelihood that the Inhuma cultivar will be more susceptible to infestation by cowpea weevils than cultivars belonging to group B, which appear to be less susceptible.

Table 3. Statistical analysis of the emerging insects number variable (Tukey test at 5% probability level).

Treatments	Repetitions	Means	Standard error	Tukey (5%)	Duncan (5%)
07-Inhuma	4	127.50	16.18	a	a
27-TE97-303G-12-BRS-Pageú	4	101.50	27.64	ab	ab
08-Pingo-de-Ouro 2	4	94.75	30.86	ab	abc
09-Pingo-de-Ouro 1-2	4	80.00	41.12	abc	abcd
20-Sempre Verde L. E. Magalhães	4	69.25	1.03	abc	abcde
25-MNC99-508G-1	4	68.25	27.35	abc	abcde
04-Paulistinha	4	68.00	28.53	abc	abcde
24-Maravilha	4	60.25	3.68	abc	abcde
26-TE 97-304G-4	4	57.50	22.71	abc	bcde
03-BRS-Marataoã	4	55.50	12.84	abc	bcde
01-IPA 207	4	51.25	5.04	abc	bcde
23-Vigna 384	4	46.75	11.80	abc	bcdef
22-Sopinha	4	44.75	11.79	abc	bcdef
16-BRS-Novaera	4	42.75	10.83	abc	bcdef
15-MNC99-519D-1-1-5	4	41.75	7.33	abc	bcdef
11-EVX91-2E-2	4	37.25	8.20	abc	bcdef
02-BRS Milênio	4	37.00	11.21	abc	bcdef
14-BRS-Potengí	4	30.25	8.94	abc	cdef
21-Rajado	4	30.00	9.28	abc	def
17-MNC99-510G-8	4	26.75	16.94	abc	def
19-Pretinho	4	26.25	3.42	abc	def
10-EVX-63-10E	4	26.00	9.59	bc	def
13-MNC00-553D-8-1-2-3	4	25.50	3.52	bc	def
18-TE96-290-12G	4	24.75	16.99	bc	def
06-Patativa	4	23.25	7.56	bc	def
05-BRS-Paraguaçu	4	23.00	5.76	bc	ef
12-MNC99-537-F4	4	16.25	10.15	c	f

In Table 2, according to the number of emerging insects for each cultivar, it is also possible to divide the data into 3 distinct groups: A, AB, and B. Again, Group A is composed only of the Inhuma cultivar, which is considered to be the most susceptible because it had a greater number of emerging insects than the others; this indicated that there is a direct correlation between the number of eggs and the number of emerging insects in this cultivar. Group AB comprises the largest number of cultivars in which there was no significant difference among the means. However, the cultivars EVX-63-10E, MNC99-510G-8, and TE96-290-12-G, which previously were scored as less susceptible to oviposition and belonged to group B, now became part of group AB, which indicates that there is no direct correlation between the number of eggs and the number of emerging insects in these cultivars. Group B is composed only of the Patativa and MNC99-537-F4 cultivars, which are considered less susceptible because they presented fewer emerging insects.

According to Barreto and Quinderé (2000), the number of eggs and the number of emerging insects were significantly and positively correlated with each other. However, according to Lara (1997), the genotypes with the most oviposition are not always the most susceptible; there may be other factors that prevent insect larval development. In this way, a genotype with heavy oviposition can still prove to be resistant.

Genetic variability

The same cultivars that were tested for resistance to *C. maculatus* were also analyzed

for genetic variability using ISSR molecular markers. Among the 10 markers that were used for the analysis, 7 (01, 842, 860, 862, 868, 878, and 886) were selected for the study according to the profiles they presented on 1.3% agarose gels. To test the primers, Sempre Verde cultivar was used, which was the same cultivar that was used as the control group in the endurance test.

In total, the 10 primers that were selected (01, 842, 860, 862, 868, 878, 886, GTG5, GACA4, and M13) produced 1337 bands. The dendrogram that was obtained by cluster analysis with the 10 primers showed great genetic variability, enough to discriminate all the cowpea cultivars at the molecular level. According to Falcão et al. (2004), when ISSR molecular markers were used in different organisms, significant differences can be observed in the amplification, which can be used to detect the polymorphism among the organisms studied and assess the differences among these organisms. Sharma et al. (2008) identified gender differences of the species *Simmondsia chinensis* (jojoba) before flowering by using ISSR markers. This identification was valuable because researchers did not have any other means to detect this difference. In order to obtain this result, they used 8 cultivars of jojoba and 42 ISSR primers. In this study, it was not possible to relate the most molecularly variable groups with the most resistant or susceptible cultivars to cowpea weevils; we determined that there was great genetic variability and distance among the most susceptible cultivars.

Abadio (2007) reviewed the grouping of ISSR data in *Phaeoisariopsis griseola* and produced a dendrogram that separated the isolates into 28 genotypes, detecting variability more efficiently than other molecular methods, including enterobacterial repetitive intergenetic consensus PCR and box sequences-polymerase chain reaction (BOX-PCR). The high level of polymorphism that was detected showed that the ISSR-PCR technique was suitable for discriminating the genotype of *P. griseola*. In our study, it was possible to observe a high level of intra-specific variation among the cultivars, whether resistant or sensitive to the cowpea weevil (75%; Tables 1 and 2), in the dendrogram that was generated by ISSR-PCR. The Inhuma cultivar was extremely susceptible to cowpea weevil attack, but in the dendrogram, it presented similarity of around 75% to the third group, which included Sopinha cultivar that features 42% resistance to cowpea weevil, and it was in a group far from Inhuma according to the resistance statistical data. Nevertheless, the genetic variability, as shown in this study, is not directly related to the resistance of the cultivars tested.

Dwivedi et al. (2001) assessed the genetic diversity of 26 accessions of peanut with 8 polymorphic primers. We used 11 primers that resulted in the formation of 3 genotypic groups, indicating that a small number of primers can generate information about the genetic variability of the 27 cultivars.

All cultivars that were evaluated presented a similarity of about 65%, which indicates significant polymorphism among the cultivars. The first group included the cultivars MNC99-508G-1, MNC99-510G-8, TE97-4, and TE97-304G-12-BRS-Pageú, which shared about 90% similarity, and the TE96-290-12G cultivar, which shared about 80% similarity with the other cultivars in this group. The second group consists of 11 cultivars, including 10 that were about 80% similar, while the cultivar MNC99-537-F4 presented approximately 75% similarity in relation to the others. In the third group, there were also 11 cultivars, including 10 that were about 80% similar, while Pretinho cultivar showed about 75% similarity with the rest of this group. It is believed that the high similarity among the cultivars resulted from the process of domestication, which contributed to a loss of genetic variability that was most pronounced by the establishment of easily detected features, such as the color and size of the seed, plant size, and type of pod (Vaillancourt et al., 1993).

Relationship between genetic variability and resistance to the cowpea weevil

When we examined the correlation between the genetic variability of cultivars and the level of susceptibility to cowpea weevil, we observed that Inhuma, which was considered to be the most susceptible based on the number of eggs and emerging insects, was about 85% similar to Sempre Verde, L.E. Magalhães, and Sopinha cultivars (Figure 1). However, these cultivars were significantly distant from Inhuma cultivar in terms of the level of susceptibility because they were part of the AB group for the two variables, indicating that there was no correlation between polymorphism and the level of susceptibility for these cultivars.

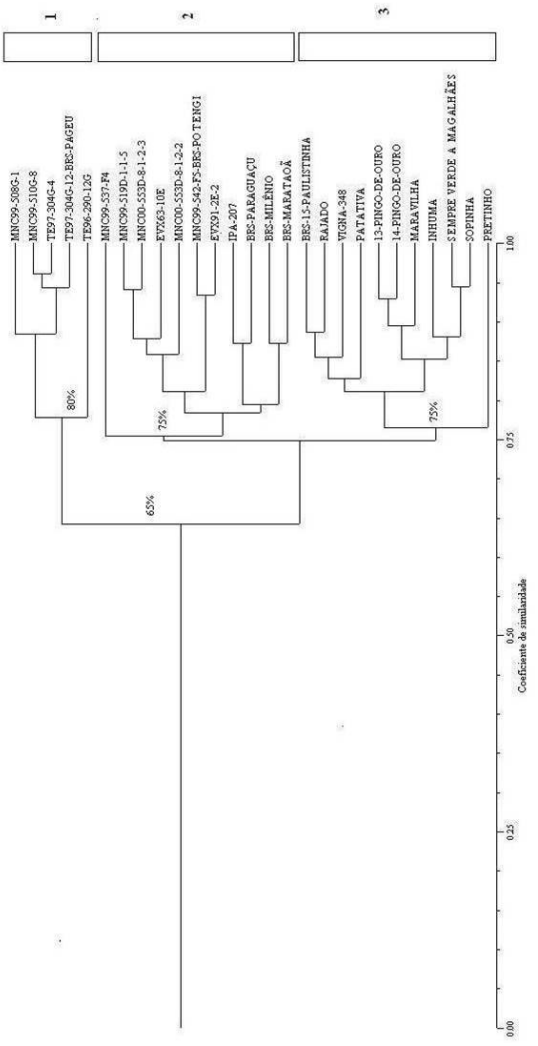

Figure 1. Dendrogram constructed by the unweighted pair group method with arithmetic average grouping method, using Jacard's coefficient of similarity and based on the molecular profiles (inter-simple sequence repeat markers) obtained from 27 cultivars of cowpea.

As for the TE96-290-12G cultivar, which was less susceptible to the number of eggs, it was approximately 80% similar to MNC99-508G-1, MNC99-510G-8, TE97-304G, and TE97-304G-12-BRS-Pageú. However, despite this great similarity, the cultivars distanced themselves significantly in the level of susceptibility to both variables, except MNC99-510G-8 cultivar, which approached TE96-290-12G for the variable of number of eggs, as shown with the Tukey test at 5%, where no significant difference was found between them.

In terms of the number of emerging insects, the cultivar MNC99-537-F4 was considered to be the least susceptible. Despite revealing about 75% similarity with the cultivars belonging to its group, there was no closeness among them in terms of the level of susceptibility according to both number of emerging insects and number of eggs. It is worthwhile to compare our study with that of Tymon and Pell (2005). In their study, 30 isolates of the entomopathogenic fungus *P. neoaphidis* from different geographic origins were analyzed using the ISSR technique; they demonstrated a high level of intra-specific variation among the isolates, but they did not identify groups that were related to the geographic origin.

According to the results that we obtained, it can be affirmed that a positive correlation is not mandatory between the levels of polymorphism and susceptibility. We observed that cultivars with great similarity in terms of genetic variability (Figure 1) distanced themselves in terms of their susceptibility to the variables that were studied.

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