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Selection of phenotypic traits and resistance to *Cowpea severe mosaic virus* and *Cowpea aphid-borne mosaic virus* in cowpea [*Vigna unguiculata* (L.) Walp.] seeds with rugose white coat

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Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important protein source in diets in Brazil. The preference of consumers is for large, white, light brown and evergreen seeds with rugose coat and small hilum and hilum ring. The *Cowpea severe mosaic virus* (CPSMV) and the *Cowpea aphid-borne mosaic virus* (CABMV) are the main pathogens in cowpea plantations in Brazil. This study selected cowpea offspring with (i) white rugose coat seed characteristics that are accepted by markets and (ii) that present resistance to CPSMV (serotype I) and CABMV. The first selection of asymptomatic plants was carried out using seedlings from F_3 seeds mechanically inoculated with a mixture of the two viruses. Offspring $F_{3:4}$ went through a second selection process with two stages, one in the field (with natural inoculation), one in trays (with mechanical inoculation). In total, 40 $F_{3:4}$ offspring were selected to evaluate agronomic traits in two field assays, one in Teresina, one in Tracuateua (states of Piauí and Pará, respectively) based on a randomized block design with four repetitions. Significant effect of genotype and of the interaction assay x genotype was observed ($p \leq 0.01$) for most of the traits evaluated: weight of 100 seeds, yield, seed length, width, and height; length-to-height ratio; width-to-height ratio, and hilum width-to-length ratio. Fourteen offspring produced large seeds (25 – 30 g in 100 seeds), and four presented cross-resistance to CPSMV and CABMV, while 36 offspring were resistant only to CPSMV.

Keywords: CABMV, CPSMV, improvement, genetic breeding, seed size.

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is native to west Africa, more specifically Nigeria, which is considered the main species diversity center (Steele and Mehra, 1980; Ng and Maréchal, 1985). Nigeria and Niger are the leading global producers of cowpea. With approximately 822,000 tons grown in 1.6 million hectares in 2011, Brazil comes third worldwide, and tops the cowpea production ranking in the Americas (Langyntuo et al., 2003; FAO, 2011). Most cowpea is produced in northeastern Brazil. The region holds record figures in the country, accounting for 68% of the production and 84% of cultivated area. The state of Ceará is the main producer, with 159,741 tons a year (MAPA, 2014).

The characterization and classification of cowpea seeds in terms of color, shape, size, and type of hilum and haulm are important tools not only in the description of cultivars, but also — and mainly — in the definition of the commercial quality of cowpea seeds. In Brazil and in nations that import cowpea, the characteristics of the hilum, of its membrane, and of the haulm are the most relevant as quality indicators for the White cowpea class. The end consumer has a preference for seeds with no haulm though with small hilum and ring, in addition to pale membranes. Seed size is yet another important phenotypic trait. For instance, domestic and foreign markets require that 100 seeds should weigh between 20 and 25 g, respectively. Besides White cowpea, light brown and evergreen seeds are also popular (Freire Filho et al., 2011a; MAPA, 2014). In like manner, a rugose coat texture is valued in both markets. Seeds of the commercial class called 'Cores' (*mulato*, *canapu*, and others) are less accepted, since seeds may become dark either before or after harvest. In light of the importance of color as a pricing parameter, dedicated improvement programs are implemented to select cultivars with high color persistence after harvest (Rocha, 2012). Viruses are the main pathogens that affect cowpea productivity (Cruz and Aragão, 2014). According to Hampton et al. (1997), cowpea is infected by eight virus species distributed in five families. In Brazil, the *Cucumber mosaic virus* (CMV), the *Cowpea aphid-borne mosaic virus* (CABMV), the *Cowpea severe mosaic virus* (CPSMV), and the *Cowpea golden mosaic virus* (CPGMV) are the most important (Lima et al., 2005). However, CPSMV and CABMV stand out as the viruses that most significantly harm cowpea crops: the former due to the severity of the infections it causes, the latter because of its prevalence (Barros et al., 2013). CPSMV, of the *Comovirus* genus, *Secoviridae* family, is transmitted semi-persistently by insects of the Chrysomelidae family (*Diabrotica speciosa* and *Cerotoma arcuata*) (Costa et al., 1978). Its bipartite genome is formed by a positive sense, single-strand RNA (of 6.0 kb and 3.7 kb) individually encapsulated in viral particles

(Sanfaçon et al., 2012). The virus induces chlorotic and necrotic spots on leaves. It also causes a severe form of mosaic, blistering and distortion of leaves, reduction of leaf blade area, and bleaching of veins. Pods and seeds may exhibit irregular spots. In addition, germination index of seeds is negatively affected. The infection sometimes causes death (Lima et al., 2005). Four CPSMV serotypes (called I, II, III and IV) have been identified in Brazil (Lin et al., 1981a; Lin et al., 1981b; Lin et al., 1984). In the state of Piauí, northeastern Brazil, only serotypes I and II were identified (Santos, 1990). CPSMV control strategies are essentially based on the growth of resistant cowpea genotypes, such as Macaibo, CNC-0434 (Assunção et al., 2005), BR-10 Piauí (Santos et al., 2000), and BR 17 (Oliveira et al., 2012). CABMV belongs to the *Potyvirus* genus, *Potyviridae* family (Adams et al., 2012). It is transmitted non-persistently by several aphid species, especially *Aphis craccivora* (Bock and Conti, 1974; Di Piero et al., 2006; Adams et al., 2012). Viral particles are elongated, flexuous, 680 to 900 nm long and measure 11 to 13 nm in diameter. The genome includes one positive sense, single-strand RNA molecule with approximately 10,000 nucleotides. CABMV infects cowpea, inducing mottle, mosaic, chlorotic spots, blisters and leaf deformation. The best control measure is based on resistant varieties (Pio Ribeiro et al., 1978), though other efforts may also be adopted such as the use of healthy seeds and the elimination of infected plants. Some genotypes have been characterized as immune to CABMV: TVu 379 (Lima et al., 1986), IT85F-2687 (Rocha et al., 2003), and TVu 966 (Oliveira et al., 2012). Studies carried out to obtain cowpea cultivars resistant to CPSMV and CABMV revealed that the resistance inherited against both viruses is monogenic recessive in character (Barros et al., 2013). This feature of the resistance pattern results from the absence of any given factor that is indispensable to viral replication or mobility inside the host (Hull, 2012).

As a contribution to the existing knowledge about quality improvements and in light of the socioeconomic importance of cowpea culture in Brazil, the present study describes the selection of *V. unguiculata* offspring with the phenotypic traits preferred in Brazilian and foreign markets, like the weight of 100 seeds (W100S) in excess of 25 g, white rugose coat with small hilum and ring and no haulm, and resistance to CPSMV and CABMV.

Results and Discussion

Evaluation of agronomic traits

Significant differences were observed in most phenotypic traits between genotypes ($p \leq 0.01$), both in the Teresina and in the Tracuateua assays. The exception was seed index, for which significant differences were observed only in the plants grown in Tracuateua, indicating the existence of genetic variability between genotypes for all traits studied in the two sites. This genetic variability is an essential factor in improvement programs, and should be appropriately exploited (Idahosa et al., 2010). Covariance analysis indicated marked genetic variability between offspring and parent plants. Genotype and the assay x genotype interaction significantly influenced all the traits evaluated ($p \leq 0.01$). The exception was the effect of the assay x genotype interaction on pod length and length-to-height ratio. The effect of the assay x genotype interaction on the seed width-to-height ratio was significant ($p \leq 0.05$). The effect of the assay x genotype interaction has been discussed by Donça (2012). The coefficient of variation (CV) was low for all traits analyzed, both in the individual and combined analyses, indicating good experimental accuracy (Pimentel Gomes, 2000).

Due to the significant interaction assay x genotype, means of each assay were analyzed individually. For W100S, six groups formed in the two locations, and several offspring reached values over 25 g, which is the recommended export standard. In Teresina, offspring P8, P15, P22, P32, and P35 had mean W100S values above 30 g, while in Tracuateua P1, P15, P32, and P35 performed better, with means around 28 g (Table 2). No offspring performed better than the large-seed parent plants, indicating that there was no allele complementarity of parent plants in this trait (Lopes et al., 2001). Table 3 shows the four groups formed in the two locations for the trait seed length. In Teresina, SL values for the offspring P8, P15, P21, and P22 were similar to those for the parent plants that produced the largest seeds (G41 and G42) and higher that the value observed for G43. In Tracuateua, the same was observed for offspring P35. As for seed width, five groups were formed in Teresina. The largest values were observed for P1, P7, P8, P15, P22, P24, P26, P27, P28, P31, and P35. In Tracuateua, six groups were formed, and offspring P15, P26, P27, and P31 had the highest seed width values. It should be highlighted that no offspring had better seed width values than large-seed parent plants. Considering seed height, two groups were formed in Teresina, four in Tracuateua. In the Teresina assay, several offspring presented identical results to those of all large-seed parent plants. In turn, in the Tracuateua assay, no offspring performed better than parent plants G41 and G42, though several had similar values as those observed for G43. The length-height relationship (J coefficient) of seeds was not influenced by the assay x genotype interaction. Two groups were formed considering the means of the two assays (Table 4). Concerning the width-to-height ratio (H coefficient), four groups were formed in Teresina. Offspring P8, P24, and P27 had similar results to those of parent plants G41 and G42, which presented the best ratios. Three groups were formed in Tracuateua, but only offspring P27 performed similarly to parent plant G41, and better than the other parent plants. Considering hilum width and length, smaller values are preferred. For the trait hilum width, four groups were formed in both environments. The best results in Teresina were observed for offspring P1, P3, P4, P5, P6, P7, P10, P13, P14, P16, P17, P18, P19, P24, P32, P37, and P39; in Tracuateua the offspring P1, P3, P5, P6, P9, P17, and P28 performed better (Table 4). Concerning hilum length, three groups were formed in Teresina, where offspring P2, P3, P5, P7, and P10 exhibited the best results. In Tracuateua, four groups of offspring were formed, with offspring P2, P5, and P10 performing better than the others. All these offspring had better values for hilum traits, when compared with parent plants G41, G42, and G43 (table 4). Mean hilum length and width values of these offspring were 3.13 and 5.35. These values are higher than those obtained by Donça (2012), 1.65 and 3.55, respectively, using parent plants that produced white seeds with short hilum. The classification of seeds in the two assays for size, form, and filling, apart from the criteria used in this classification are shown in Table 5. In Teresina, 43% of genotypes presented large seeds, 43% produced midsized seeds, 12% had extra-large seeds, 2% had small to midsized seeds. In Tracuateua, 49% of seeds were mid-to-large, 37% were large, 12% were mid-to-small, and 2% were extra-large. These results are promising, since both domestic and international cowpea markets prefer large and extra-large seeds (Freire Filho et al., 2011a). Despite being low, the percentage of genotypes with extra-large, white and rugose seeds represents a breakthrough in cowpea genetic

Table 1. Parent plants and crossings used to obtain offspring. Description of parent plants from the cowpea germplasm bank of Embrapa Meio-Norte and of the crossings used to obtain offspring in this study.

	Trait			
Parent plant	Seed	Size	Plant	Resistance to CPSMV and CABMV
MNC08-928E-11J	White	Midsize to large	Semi-upright	Highly resistant
MNC05-828C-3-15-1	White	Large	Semi-upright	Susceptible
MNC05-828C-3-15-2	White	Large	Semi-upright	Susceptible
MNC05-828C-2-1-1	White	Large	Semi-upright	Susceptible
Offspring	Crossings			
MNC11–1071	MNC08-928E-11J x MNC05-828C-3-15-1			
MNC11–1072	MNC08-928E-11J x MNC05-828C-3-15-2			
MNC11–1073	MNC05-828C-2-1-1 x MNC08-928E-11J			

improvement programs, since no accesses with these characteristics are present in the germplasm bank of the cowpea genetic improvement program of the Federal University of Ceará (Paiva et al., 2014), and rare are the accesses in a similar program maintained by Embrapa Meio-Norte (Freire Filho et al., 2011b).

Puerta Romero (1961) conceived two coefficients, J and H, to characterize seed shape and seed filling. In the present study, six and three types of seeds were observed in Teresina and Tracuateua, respectively. Approximately 80% of genotypes in both assays produced elliptical seeds, which is a shape that is gaining wider preference in the market. In Teresina, offspring P8 and P9 produced short, kidney-shaped seeds, which is one of the aims of cowpea improvement programs. Regarding seed filling, 72% of seeds obtained in Teresina were semi-filled seeds, while 28% were filled seeds. In Tracuateua 95% and 5% of genotypes were semi-filled and filled, respectively. The filled type is prefered, and is the object of cowpea improvement programs (Mishili et al., 2009).

Evaluation of resistance to viruses

Visual evaluation of cowpea seeds, carried out 40 days after seeding, and the results of the molecular and serological assays used to evaluate resistance to CPSMV and CAMBV of the 40 offspring (P1 to P40) and of the four parent plants (G41 to G44) that were mechanically inoculated with the viruses are shown in Table 6. All offspring selected were resistant to CPSMV both in the visual inspection and in the RT-PCR assays. As expected, parent plants G41, G42, and G43 were highly susceptible to CPSMV, while G44 was resistant. RT-PCR of G41 and G42 afforded to amplify a 592-bp fragment, indicating the infection with CPSMV (data not shown). The offspring and parent plant G44 did not amplify this fragment, pointing to resistance to the virus. Several CPSMV-resistant cowpea strains have been identified (Lima et al., 1986; Santos, 1990; Barreto and Santos, 1999; Passos, 1999; Paz et al., 1999; Oliveira, 2012). However, these strains do not produce large, rugose seeds, upright plants, and therefore do not guarantee high commercial value. The two evaluations of the resistance to CABMV showed that parent plant G44, which confers resistance to both virus, selected resistance to the virus, which explains the high number of offspring with symptoms of CABMV infection (Table 6). This selection is a result of natural, unexpected crossings during parent plant seed propagation. Offspring P3, P12, P17, and P24 were resistant to CABMV. In turn, P1, P2, P5, P6, P13, P14, P19, P20, P23, P25, P28, P29, P34, and P38 presented similar amounts of resistant and susceptible individuals, which justify a new selection for resistance to the virus. Offspring P5, P7, P8, P9, P11, P15, and P27 were moderately resistant, since they presented only mild mosaic. The other offspring were highly resistant and did not express symptoms in the field evaluations. Several resistant cowpea strains have been identified (Lima et al., 1986; Barreto and Santos, 1999; Rocha et al., 2003; Oliveira et al., 2012; Barros et al., 2013), none of which presents the agronomic characters exhibited by the offspring selected in the present study. Four offspring were resistant to both viruses (P3, P12, P17, and P24) (Table 6). This cross-resistance had been identified in other cowpea genotypes (Oliveira et al., 2012; Barros et al., 2013), but these genotypes are not correlated with the seed traits obtained in the present selection effort.

Synergism between CPSMV and CABMV (Table 6) was also observed in parent plants G41, G42, and G43, which caused apical death in all these individuals. The specialized literature cites studies that demonstrated the synergistic and antagonistic effects of double viral infections in several pathological systems (Wang et al., 2002; García-Cano et al., 2006; Martín and Elena, 2009). However, few studies have addressed this topic in cowpea (Pio-Ribeiro et al., 1978; Kareem and Taiwo, 2007; Taiwo et al., 2007), though it is known that, in more severe viral infections, synergistic effects as a whole are always associated with the presence of a potyvirus (Kareem and Taiwo, 2007; Pio-Ribeiro et al., 1978). Taken together, such findings underscore the importance of the present study, since parent plants, despite their desired phenotypic traits, are highly vulnerable to viruses, and offspring perform better than their parent plants in terms of resistance to at least one virus.

Materials and Methods

Plant material

Parent plants were from the germplasm cowpea bank of Embrapa Meio-Norte, Brazil: (i) MNC08-928E-11-J, with small seeds and hilum, highly resistant to CPSMV and CABMV, and (ii) MNC05-828C-3-15-1, MNC05-828C-3-15-2, and MNC05-828C-2-1-1, which have large seeds and hilum, excellent commercial quality, and increased susceptibility to CPSMV and CABMV. All parent plants are semi-erect, have white seeds, and no haulm. A brief description of the parent plants is given in Table 1.

Maintenance of viral isolates in cowpea marker plants

The viruses used in the present study were obtained from cowpea plants naturally infected in experimental fields managed by Embrapa Meio-Norte. After virus isolation,

Table 2. Mean values of cowpea productivity factors and productivity (pod length, PL; number of seed per pod, NSP; weight of 100 seeds, W100S) in two locations in Brazil (Teresina, state of Piauí, and Tracuateua, state of Pará).

Offspring		Trait											
Nº	Code	PL (cm)				NSP (unit)				W100S (g)			
		Teresina	Tracua-teua	Mean		Teresina		Tracua-teua		Teresina		Tracua-teua	
1	MNC11-1071B-2	19.40	18.50	18.95	a	12.45	b	10.94	b	28.52	d	28.02	b
2	MNC11-1071B-19	18.30	17.25	17.78	b	9.50	d	7.35	d	23.82	e	23.82	d
3	MNC11-1071B-20	19.10	18.70	18.90	a	12.10	b	11.73	a	21.92	f	21.85	e
4	MNC11-1071B-22	17.45	17.60	17.53	b	12.15	b	11.02	b	23.77	e	22.60	d
5	MNC11-1071B-38	17.71	17.80	17.76	b	9.79	d	9.20	c	24.20	e	21.92	e
6	MNC11-1071B-43	19.35	18.60	18.98	a	11.76	c	8.82	c	27.05	d	25.77	c
7	MNC11-1071B-44	18.40	18.40	18.40	a	10.43	c	8.95	c	25.40	e	23.05	d
8	MNC11-1071B-46	18.30	18.80	18.55	a	8.60	e	8.56	c	30.72	c	27.40	c
9	MNC11-1071B-56	19.20	17.35	18.28	a	10.91	c	8.20	d	23.72	e	22.97	d
10	MNC11-1071B-57	17.95	17.15	17.55	b	12.27	b	10.20	b	22.47	f	23.12	d
11	MNC11-1071B-60	17.95	17.35	17.65	b	11.01	c	8.55	c	23.80	e	22.75	d
12	MNC11-1071B-61	18.81	18.40	18.61	a	10.66	c	9.75	c	23.62	e	19.75	f
13	MNC11-1071B-62	19.10	19.70	19.40	a	11.42	c	11.15	b	22.45	f	20.77	f
14	MNC11-1071B-118	17.75	16.85	17.30	b	9.20	d	8.17	d	25.15	e	25.15	d
15	MNC11-1071B-121	18.55	18.60	18.58	a	8.15	e	8.00	d	29.55	c	28.67	b
16	MNC11-1071B-122	19.00	19.10	19.05	a	12.65	b	10.93	b	21.50	f	21.92	e
17	MNC11-1071B-123	19.00	17.85	18.43	a	14.50	a	12.05	a	19.37	f	20.00	f
18	MNC11-1071B-126	18.60	19.20	18.90	a	11.04	c	10.45	b	26.52	e	26.87	c
19	MNC11-1071B-127	18.15	18.30	18.23	a	8.25	e	11.96	a	24.70	e	21.45	e
20	MNC11-1072B-134	18.55	18.20	18.38	a	11.15	c	10.00	b	24.20	e	23.67	d
21	MNC11-1072B-139	19.20	18.90	19.05	a	8.70	e	9.17	c	27.70	d	25.92	c
22	MNC11-1072B-183	19.30	19.20	19.25	a	7.50	e	7.16	d	31.42	c	29.80	b
23	MNC11-1072B-194	18.20	18.25	18.23	a	9.80	d	9.65	c	23.65	e	19.62	f
24	MNC11-1073B-206	17.60	17.30	17.45	b	10.65	c	9.70	c	25.90	e	22.22	e
25	MNC11-1073B-212	19.15	17.90	18.53	a	12.17	b	10.65	b	24.10	e	20.35	f
26	MNC11-1073B-214	17.00	16.80	16.90	b	8.40	e	7.90	d	26.92	d	26.57	c
27	MNC11-1073B-216	17.05	16.75	16.90	b	9.65	d	9.85	b	26.92	d	24.02	d
28	MNC11-1073B-219	17.90	16.90	17.40	b	8.75	e	7.35	d	25.87	e	24.67	d
29	MNC11-1073B-226	17.50	18.30	17.90	b	7.80	e	9.10	c	27.97	d	25.15	d
30	MNC11-1073B-227	18.15	17.45	17.80	b	12.05	b	9.25	c	25.82	e	23.65	d
31	MNC11-1073B-230	16.85	16.45	16.65	b	9.80	d	9.10	c	27.57	d	27.22	c
32	MNC11-1073B-232	17.75	17.80	17.78	b	11.55	c	9.64	c	24.60	e	28.27	b
33	MNC11-1073B-233	17.80	17.10	17.45	b	10.05	d	8.90	c	28.72	d	26.12	c
34	MNC11-1073B-234	17.15	16.85	17.00	b	10.05	d	9.30	c	27.32	d	25.97	c
35	MNC11-1073B-235	19.15	19.75	19.45	a	9.11	d	10.20	b	30.37	c	28.07	b
36	MNC11-1073B-237	19.30	17.95	18.63	a	11.75	c	9.35	c	23.67	e	22.00	e
37	MNC11-1073B-243	17.45	17.20	17.33	b	8.65	e	8.05	d	25.10	e	23.85	d
38	MNC11-1073B-246	18.21	17.35	17.78	b	11.35	c	9.50	c	25.37	e	24.07	d
39	MNC11-1073B-253	17.25	16.70	16.98	b	9.86	d	9.40	c	21.90	f	19.97	f
40	MNC11-1073B-256-1	17.35	17.95	17.65	b	10.75	c	10.65	b	21.60	f	18.65	f
41	MNC05-828C-3-15-1	19.50	19.45	19.48	a	9.15	d	8.85	c	38.47	a	36.80	a
42	MNC05-828C-3-15-2	18.85	18.85	18.85	a	7.56	e	7.20	d	34.65	b	35.30	a
43	MNC05-829C-2-1-1	18.29	17.65	17.97	b	11.55	c	8.85	c	29.37	c	29.70	b
44	MNC08-928E-11-J	17.00	16.35	16.68	b	10.15	d	9.60	c	21.25	f	21.47	e
Means of offspring		18.25	17.91	18.08		10.41		9.50		25.37		23.94	
Means of parent plants		18.41	18.08	18.24		9.60		8.63		30.94		30.82	
Global mean		18.26	17.93	18.10		10.34		9.42		25.88		24.57	

Means in the same column that are followed by identical lowercase letters indicate no statistically significant difference using the Scott-Knott test ($p>0.05$).

Table 3. Mean values of cowpea seed traits (seed length, SL; seed width, SW; and seed height, SH) in two locations in Brazil (Teresina, state of Piauí, and Tracuateua, state of Pará).

Offspring		Trait											
Nº	Code	SL (mm)				SW (mm)				SH (mm)			
		Teresina		Tracua-teua		Teresina		Tracua-teua		Teresina		Tracua-teua	
1	MNC11-1071B-2	10.78	c	10.49	c	5.96	c	5.69	d	7.48	a	7.39	b
2	MNC11-1071B-19	10.95	c	10.81	c	5.22	e	5.48	e	7.26	b	7.52	b
3	MNC11-1071B-20	10.63	c	10.17	d	5.65	d	5.16	f	7.32	a	7.14	c
4	MNC11-1071B-22	9.72	d	10.24	d	5.27	e	5.53	e	6.92	b	7.57	b
5	MNC11-1071B-38	10.92	c	10.04	d	5.24	e	5.07	f	7.21	b	7.10	c
6	MNC11-1071B-43	10.69	c	11.05	b	5.56	d	5.77	d	7.26	b	7.54	b
7	MNC11-1071B-44	10.84	c	10.73	c	5.77	c	5.36	e	7.13	b	7.22	c
8	MNC11-1071B-46	11.88	a	11.35	b	6.02	c	5.66	d	7.17	b	7.48	b
9	MNC11-1071B-56	10.26	d	10.65	c	5.65	d	5.54	e	7.03	b	7.26	c
10	MNC11-1071B-57	9.88	d	10.05	d	5.33	e	5.15	f	7.04	b	7.05	d
11	MNC11-1071B-60	11.28	b	10.46	c	5.68	d	5.22	f	7.41	a	7.27	c
12	MNC11-1071B-61	10.76	c	10.35	d	5.41	e	4.93	f	6.84	b	7.04	d
13	MNC11-1071B-62	10.98	c	10.51	c	5.43	e	5.30	f	7.00	b	7.04	d
14	MNC11-1071B-118	10.74	c	10.17	d	5.51	d	5.53	e	7.42	a	7.44	b
15	MNC11-1071B-121	11.96	a	11.40	b	5.89	c	5.91	c	7.43	a	7.64	b
16	MNC11-1071B-122	10.41	c	10.74	c	5.09	e	5.38	e	6.95	b	7.49	b
17	MNC11-1071B-123	10.01	d	9.74	d	5.24	e	5.01	f	6.80	b	6.81	d
18	MNC11-1071B-126	11.21	b	10.69	c	5.64	d	5.56	e	7.63	a	7.60	b
19	MNC11-1071B-127	11.22	b	10.70	c	5.70	d	5.12	f	7.55	a	7.10	c
20	MNC11-1072B-134	10.54	c	10.96	c	5.43	e	5.31	f	6.99	b	7.22	c
21	MNC11-1072B-139	11.76	a	10.81	c	5.45	e	5.49	e	6.91	b	7.15	c
22	MNC11-1072B-183	11.81	a	11.66	b	5.94	c	5.67	d	7.23	b	7.57	b
23	MNC11-1072B-194	11.18	b	11.10	b	5.42	e	5.21	f	7.26	b	7.21	c
24	MNC11-1073B-206	10.86	c	10.28	d	5.85	c	5.64	d	6.99	b	7.07	c
25	MNC11-1073B-212	11.24	b	10.44	c	5.62	d	5.27	f	7.20	b	6.92	d
26	MNC11-1073B-214	11.38	b	11.15	b	5.97	c	5.88	c	7.40	a	7.49	b
27	MNC11-1073B-216	10.89	c	10.85	c	5.93	c	5.88	c	7.10	b	6.89	d
28	MNC11-1073B-219	10.51	c	10.72	c	5.82	c	5.58	e	7.47	a	7.53	b
29	MNC11-1073B-226	11.35	b	11.15	b	5.67	d	5.70	d	7.72	a	7.35	b
30	MNC11-1073B-227	10.44	c	10.54	c	5.55	d	5.42	e	7.15	b	7.24	c
31	MNC11-1073B-230	10.94	c	11.02	b	6.05	c	5.88	c	7.55	a	7.51	b
32	MNC11-1073B-232	10.02	d	10.54	c	5.63	d	5.73	d	7.03	b	7.27	c
33	MNC11-1073B-233	10.37	c	9.99	d	5.69	d	5.78	d	7.48	a	7.49	b
34	MNC11-1073B-234	10.45	c	10.04	d	5.62	d	5.55	e	7.74	a	7.59	b
35	MNC11-1073B-235	11.39	b	12.13	a	5.87	c	5.66	d	7.49	a	7.54	b
36	MNC11-1073B-237	10.74	c	10.70	c	5.05	e	5.55	e	7.16	b	7.45	b
37	MNC11-1073B-243	10.39	c	10.68	c	5.45	e	5.45	e	7.16	b	7.32	c
38	MNC11-1073B-246	10.06	d	10.67	c	5.38	e	5.55	e	7.09	b	7.29	c
39	MNC11-1073B-253	10.71	c	10.39	c	5.69	d	5.49	e	7.26	b	7.00	d
40	MNC11-1073B-256-1	10.98	c	10.35	d	5.30	e	5.08	f	6.99	b	6.70	d
41	MNC05-828C-3-15-1	12.31	a	12.60	a	6.58	a	6.67	a	7.59	a	7.98	a
42	MNC05-828C-3-15-2	12.14	a	12.14	a	6.53	a	6.33	b	7.60	a	7.99	a
43	MNC05-829C-2-1-1	11.45	b	11.71	b	6.22	b	6.08	c	7.57	a	7.69	b
44	MNC08-928E-11-J	9.75	d	9.82	d	5.38	e	5.40	e	6.88	b	7.12	c
Means of offspring		10.83		10.66		5.59		5.48		7.23		7.29	
Means of parent plants		11.41		11.57		6.18		6.12		7.41		7.70	
Global mean		10.88		10.75		5.64		5.54		7.25		7.32	

Means in the same column that are followed by identical lowercase letters indicate no statistically significant difference using the Scott-Knott test ($p>0.05$).

Table 4 Mean values of seed traits (seed length-to-height ratio, SLSH; seed width-to- height ratio, SWSL; hilum length, HL; hilum width, HW) in two locations in Brazil (Teresina, state of Piauí, and Tracuateua, state of Pará).

Offspring Nº	Code	Trait															
		SLSH (mm)				SWSL (mm)				HW (mm)				HL (mm)			
		Teresina	Tracua-teua	Mean		Teresina		Tracua-teua		Teresina		Tracua-teua		Teresina		Tracua-teua	
1	MNC11-1071B-2	1.44	1.46	1.45	b	0.79	b	0.77	b	3.07	a	3.08	a	5.72	c	5.35	b
2	MNC11-1071B-19	1.51	1.43	1.47	b	0.71	d	0.72	c	3.29	c	3.44	c	4.88	a	4.95	a
3	MNC11-1071B-20	1.45	1.42	1.44	b	0.77	c	0.72	c	3.05	a	3.09	a	5.09	a	5.42	b
4	MNC11-1071B-22	1.40	1.35	1.38	b	0.76	c	0.73	c	2.86	a	3.27	b	5.26	b	5.56	c
5	MNC11-1071B-38	1.51	1.41	1.46	b	0.72	d	0.71	c	3.04	a	3.15	a	5.05	a	5.20	a
6	MNC11-1071B-43	1.47	1.46	1.47	b	0.76	c	0.76	b	3.08	a	3.13	a	5.19	b	5.65	c
7	MNC11-1071B-44	1.52	1.48	1.50	a	0.80	b	0.74	c	3.09	a	3.22	b	5.03	a	5.40	b
8	MNC11-1071B-46	1.66	1.51	1.59	a	0.84	a	0.75	c	3.18	b	3.31	b	5.59	c	5.85	d
9	MNC11-1071B-56	1.45	1.46	1.46	b	0.80	b	0.76	b	3.13	b	3.14	a	5.18	b	5.56	c
10	MNC11-1071B-57	1.40	1.42	1.41	b	0.75	c	0.73	c	3.06	a	3.21	b	5.05	a	5.06	a
11	MNC11-1071B-60	1.52	1.43	1.48	b	0.76	c	0.71	c	3.22	c	3.44	c	5.25	b	5.35	b
12	MNC11-1071B-61	1.57	1.47	1.52	a	0.79	b	0.70	c	3.14	b	3.28	b	5.53	c	5.55	c
13	MNC11-1071B-62	1.56	1.49	1.53	a	0.77	c	0.75	c	3.04	a	3.33	b	5.31	b	5.50	b
14	MNC11-1071B-118	1.44	1.36	1.40	b	0.74	d	0.74	c	3.04	a	3.22	b	5.27	b	5.51	b
15	MNC11-1071B-121	1.61	1.49	1.55	a	0.79	b	0.77	b	3.13	b	3.33	b	5.20	b	5.38	b
16	MNC11-1071B-122	1.50	1.43	1.47	b	0.73	d	0.71	c	3.04	a	3.47	c	5.27	b	5.65	c
17	MNC11-1071B-123	1.47	1.43	1.45	b	0.77	c	0.73	c	2.89	a	2.94	a	5.30	b	5.26	b
18	MNC11-1071B-126	1.47	1.40	1.44	b	0.74	d	0.73	c	2.98	a	3.42	c	5.39	b	5.38	b
19	MNC11-1071B-127	1.48	1.50	1.49	a	0.75	c	0.72	c	3.05	a	3.32	b	5.34	b	5.45	b
20	MNC11-1072B-134	1.51	1.52	1.52	a	0.77	c	0.73	c	3.15	b	3.24	b	5.32	b	5.68	c
21	MNC11-1072B-139	1.70	1.51	1.61	a	0.79	b	0.76	b	3.22	c	3.40	c	5.61	c	5.45	b
22	MNC11-1072B-183	1.63	1.54	1.59	a	0.82	b	0.74	c	3.12	b	3.50	c	5.55	c	5.73	c
23	MNC11-1072B-194	1.54	1.54	1.54	a	0.74	d	0.72	c	3.16	b	3.19	b	5.37	b	5.68	c
24	MNC11-1073B-206	1.55	1.45	1.50	a	0.83	a	0.79	b	3.02	a	3.23	b	5.66	c	5.78	c
25	MNC11-1073B-212	1.56	1.51	1.54	a	0.78	c	0.76	b	3.40	c	3.38	c	5.75	c	5.71	c
26	MNC11-1073B-214	1.53	1.48	1.51	a	0.80	b	0.78	b	3.27	c	3.37	c	5.49	c	6.13	d
27	MNC11-1073B-216	1.53	1.54	1.54	a	0.83	a	0.85	a	3.21	c	3.31	b	5.32	b	5.78	c
28	MNC11-1073B-219	1.41	1.42	1.42	b	0.78	c	0.74	c	3.18	b	3.16	a	5.21	b	6.64	c
29	MNC11-1073B-226	1.47	1.51	1.49	a	0.73	d	0.77	b	3.23	c	3.44	c	5.77	c	5.79	c
30	MNC11-1073B-227	1.46	1.46	1.46	b	0.77	c	0.74	c	3.27	c	3.37	c	5.31	b	5.56	c
31	MNC11-1073B-230	1.45	1.47	1.46	b	0.80	b	0.78	b	3.29	c	3.67	d	5.50	c	5.88	d
32	MNC11-1073B-232	1.42	1.45	1.44	b	0.80	b	0.78	b	3.00	a	3.30	b	5.30	b	5.74	c
33	MNC11-1073B-233	1.38	1.33	1.36	b	0.76	c	0.77	b	3.24	c	3.53	c	5.31	b	5.36	b
34	MNC11-1073B-234	1.35	1.32	1.34	b	0.72	d	0.73	c	3.27	c	3.44	c	5.59	c	5.74	c
35	MNC11-1073B-235	1.52	1.60	1.56	a	0.78	c	0.75	c	3.14	b	3.43	c	5.39	b	6.12	d
36	MNC11-1073B-237	1.50	1.46	1.48	b	0.70	d	0.74	c	3.17	b	3.34	b	5.16	b	5.65	c
37	MNC11-1073B-243	1.45	1.46	1.46	b	0.76	c	0.74	c	3.04	a	3.25	b	5.27	b	5.29	b
38	MNC11-1073B-246	1.45	1.46	1.46	b	0.75	c	0.76	b	3.17	b	3.34	b	5.31	b	5.80	c

39	MNC11-1073B-253	1.47	1.48	1.48	b	0.78	c	0.78	b	3.05	a	3.22	b	5.47	c	5.59	c
40	MNC11-1073B-256-1	1.58	1.54	1.56	a	0.75	c	0.75	c	3.18	b	3.26	b	5.62	c	5.75	c
41	MNC05-828C-3-15-1	1.62	1.57	1.60	a	0.86	a	0.83	a	3.58	d	3.84	d	5.63	c	5.97	d
42	MNC05-828C-3-15-2	1.60	1.51	1.56	a	0.86	a	0.79	b	3.72	d	3.77	d	5.60	c	5.98	d
43	MNC05-829C-2-1-1	1.47	1.52	1.50	a	0.82	b	0.79	b	3.34	c	3.51	c	5.52	c	5.70	c
44	MNC08-928E-11-J	1.41	1.38	1.40	b	0.78	c	0.75	c	3.06	a	3.29	b	4.92	a	5.28	b
Means of offspring		1.50	1.46	1.48		0.77		0.75		3.13		3.30		5.35		5.60	
Means of parent plants		1.53	1.50	1.51		0.83		0.79		3.43		3.60		5.42		5.73	
Global mean		1.50	1.46	1.48		0.78		0.75		3.16		3.33		5.36		5.61	

Means in the same column that are followed by identical lowercase letters indicate no statistically significant difference using the Scott-Knott test (p>0.05).

Table 5. Seed size and shape classification of offspring and parent plants in two locations in Brazil (Teresina, state of Piauí, and Tracuateua, state of Pará).

Seed shape and size (1)	Classes	Number of genotypes	
		Teresina	Tracuateua
Weigh of 100 seeds			
Extra small	< 10 g		
Small	10.1 to 15 g		
Midsize to small	15.1 to 20 g	1	5
Midsize to large	20.1 to 25 g	19	21
Large	25.1 to 30 g	19	16
Extra-large	> 30 g	5	2
J coefficient (J = C/A)			
Spherical	1.16 to 1.42	7	10
Elliptical	1.43 to 1.65	35	34
Kidney shaped, short	1.66 to 1.85	2	
Kidney shaped, midsized	1.86 to 2.00		
Kidney shaped, long	> 2.00		
H coefficient (H = L/A)			
Flat	< = 0.69		
Semi-filled	0.70 to 0.79	32	42
Filled	> = 0.80	12	2

(1) Seed size according to Freire Filho et al. (2012) and seed shape as described by Puerta Romero (1961).

Table 6. Results of the visual inspection and RT-PCR and PTA-ELISA in cowpea offspring mechanically inoculated with CPSMV and CABMV to evaluate resistance to these viruses in Teresina, Brazil.

Nº	Offspring	CPSMV			CABMV			CPSMV[1]	CABMV[2]
		Visual inspection			Visual inspection			RT-PCR[3]	ELISA[3]
		N° Asymptomatic plants	N° Symptomatic plants	Symptoms[4]	N° Asymptomatic plants	N° Symptomatic plants	Symptoms[4]		
1	MNC11-1071B-2	8	0	As	4	4	MM	–	±
2	MNC11-1071B-19	8	0	As	7	1	MM	–	±
3	MNC11-1071B-20	8	0	As	8	0	As	–	–
4	MNC11-1071B-22	8	0	As	5	3	MM	–	+
5	MNC11-1071B-38	8	0	As	3	5	MM	–	±
6	MNC11-1071B-43	8	0	As	4	4	MM	–	±
7	MNC11-1071B-44	8	0	As	4	4	MM	–	+
8	MNC11-1071B-46	8	0	As	6	2	MM	–	+
9	MNC11-1071B-56	8	0	As	5	3	MM	–	+
10	MNC11-1071B-57	8	0	As	0	8	Mo	–	+
11	MNC11-1071B-60	8	0	As	6	2	MM	–	+
12	MNC11-1071B-61	8	0	As	8	0	As	–	–
13	MNC11-1071B-62	8	0	As	6	2	MM	–	±
14	MNC11-1071B-118	8	0	As	4	4	Mo	–	±
15	MNC11-1071B-121	8	0	As	4	4	MM	–	+
16	MNC11-1071B-122	8	0	As	0	8	Mo	–	+
17	MNC11-1071B-123	8	0	As	8	0	As	–	–
18	MNC11-1071B-126	8	0	As	0	8	Mo	–	+
19	MNC11-1071B-127	8	0	As	3	5	Mm	–	±
20	MNC11-1072B-134	8	0	As	5	3	MM	–	±
21	MNC11-1072B-139	8	0	As	3	5	Mo	–	+
22	MNC11-1072B-183	8	0	As	2	6	Mo	–	+

(Continued)

°	Offspring	CPSMV			CABMV			CPSMV[1]	CABMV[2]
		Visual inspection			Visual inspection			RT-PCR[3]	ELISA[3]
		N° Asymptomatic plants	N° Symptomatic plants	Symptoms[4]	N° Asymptomatic plants	N° Symptomatic plants	Symptoms [4]		
23	MNC11-1072B-194	8	0	As	7	1	MM	─	±
24	MNC11-1073B-206	8	0	As	8	0	As	─	─
25	MNC11-1073B-212	8	0	As	6	2	MM	─	±
26	MNC11-1073B-214	8	0	As	0	8	Mo	─	+
27	MNC11-1073B-216	8	0	As	2	6	MM	─	+
28	MNC11-1073B-219	8	0	As	4	4	Mo, Bl	─	±
29	MNC11-1073B-226	8	0	As	5	3	MM	─	±
30	MNC11-1073B-227	8	0	As	1	7	Mo	─	+
31	MNC11-1073B-230	8	0	As	2	6	Mo	─	+
32	MNC11-1073B-232	8	0	As	1	7	Mo	─	+
33	MNC11-1073B-233	8	0	As	0	8	MM	─	+
34	MNC11-1073B-234	8	0	As	5	3	Mo	─	±
35	MNC11-1073B-235	8	0	As	2	6	Mo	─	+
36	MNC11-1073B-237	8	0	As	0	8	Mo	─	+
37	MNC11-1073B-243	8	0	As	2	6	Mo	─	+
38	MNC11-1073B-246	8	0	As	0	8	MM	─	±
39	MNC11-1073B-253	8	0	As	1	7	MM	─	+
40	MNC11-1073B-256-1	8	0	As	0	8	Mo, Bl	─	+
41	MNC05-828C-3-15-1	0	8	SM, LR, Bl, AD	0	8	SM, LR, Bl, AD	+	+
42	MNC05-828C-3-15-2	0	8	SM, LR, Bl, AD	0	8	SM, LR, Bl, AD	+	+
43	MNC05-829C-2-1-1	0	8	SM, LR, Bl, AD	0	8	SM, LR, Bl, AD	+	+
44	MNC08-928E-11-J	8	0	AS	4	4	Mo	─	±

[1] *Cowpea severe mosaic virus* serotype I; [2] *Cowpea aphid-borne mosaic virus*; [3] (+) = positive samples; (─) negative samples; (±) positive and negative samples [4] Evaluation of symptoms 40 days after seeding: As: asymptomatic; SM: severe mosaic; Mo: mosaic; MM: mild mosaic; Bl: blisters; LR: leaf reduction; AD: apical death.

mechanical inoculation and serological and molecular assays were carried out. Then, viral isolates CPSMV (serotype I) and CABMV were maintained in marker cultivars TE93-200-49F and PAMPO, respectively, in different greenhouses protected with anti-aphid screens throughout the experimental period. These isolates were used as source of inoculum during the mechanical infection of plants in all stages of this study.

Mechanical inoculation with viral isolates

Leaf extracts were prepared with 500 mg of leaves of cultivar TE93-200-49F experimentally infected with CPSMV, and 500 g of leaves of cultivar Pampo infected with CABM in sodium phosphate buffer 0.01 M, pH 70.0 at 1:10 (g/mL) using a sterilized china mortar and pestle. Mechanical inoculation was carried out scrubbing the extract on the adaxial side of leaves previously sprinkled with Celite abrasive (Sigma). Inoculations were carried out using leaf extract at 15ºC. After inoculation, plants were kept in a greenhouse protected by an anti-aphid net and controlled temperature (25ºC) and relative humidity (85%).

Serological and molecular assays

The 40 offspring selected and the four individuals mechanically inoculated were analyzed using the plate-trapped antigen - enzyme linked immunosorbent assay (PTA-ELISA) with specific anti-CABMV polyclonal antiserum, and the RT-PCR protocol (using specific primers for CPSMV) according to Barros et al. (2013). Absorbance was read at 405 nm in an ELISA reader (Microplate Reader 3550-UV, Bio-Rad) in triplicates, after the application of p-nitrophenylphosphate as substrate. The results obtained were expressed as the ratio of mean absorbance of samples infected to mean absorbance of healthy samples (negative controls). Samples were considered positive when mean absorbance readings were at least three times as high as negative control absorbance values (Barros et al., 2013). Total RNA was extracted from 0.1 g cowpea leaf tissue in Trizol^{TM} medium (Life Technologies) according to the manufacturer's instructions. RT-PCR was conducted as described by Barros et al. (2013) using approximately 1 μg total RNA and specific primers designed to amplify the protein coat gene of CPSMV (antisense: 5'-CTCAAACCCCTGTTGGGACCACA-3'; sense: 5'-GGATGAATTTTTGATGGCATGG - 3'). Samples were then placed in a thermocycler and, after an initial heating at 94^{o}C for 5 min, the amplification was conducted as follows: 30 cycles at 94^{o}C for 1 min, followed by 47^{o}C for 2 min and 72^{o}C for 3 min, and a final extension at 72^{o}C for 7 min. The size of the PCR product expected was 592 bp. Amplified DNA fragments were visualized on agarose gels 1.2%, in presence of ethidium bromide, under ultraviolet light (Sambrook et al., 1989).

Selection for large seeds

Seeds of F_3 offspring obtained from the offspring MNC-11-1071, MNC11-1072 and MNC11-1073 were selected for size. Seeds were sifted through a 0.8-mm mesh sieve. Only retained seeds were used. The pairs of parent plants that produced the three offspring are shown in Table 1.

First selection of plants with no symptoms of viral infection

The first selection of F_3 plants with no symptoms of viral infection was carried out in two steps. The first was conducted in planting trays, the second occurred after replanting in the field. Thirty-two styropor trays with 128 cells each were used. In the seeding stage, 1,024 individuals of each offspring were inoculated, totaling 3,072 plants. Eight cells were used for each offspring and each parent plant. Two seeds of each offspring were planted in eight separate cells. The same procedure was conducted for parent seeds. Trays were inspected after five days. When the two seeds germinated, one seedling was removed so that each offspring and parent plant were represented by one seedling only. The four parent plants were seeded in alternate cells with offspring. However, only four parent plants were inoculated. The other four were used as control. Mechanical inoculations were carried out six days after seeding. A second inoculation procedure occurred four days later. The main symptoms of viral infection considered as exclusion criteria were blisters (Bl), mosaic (Mo), mild mosaic (Mm), severe mosaic (Sm), leaf reduction (Lr), leaf deformation (Ld), and apical death (Ad). The plants that did not exhibit symptoms were replanted on the field, where they were exposed to natural inoculation. This selection process in the field considered the same exclusion criteria as adopted in the exclusion of individuals in the greenhouse.

Second selection of plants without symptoms

Offspring $F_{3:4}$ were submitted to a second selection process, also conducted in two stages. The first included a field study conducted according to an augmented block design with 260 offspring, for each of which the following parameters were analyzed (i) the number of plants with and without symptoms after spontaneous inoculation, (ii) the number of days before flowering started, (iii) the weight of 100 seeds, and (iv) seed yield. In the second stage the remaining F3:4 offspring selected in the field were submitted to mechanical inoculation in trays using a mixture of CABMV and CPSMV. In this stage, offspring that presented at least one symptom were excluded. The others were replanted for seed multiplication.

Evaluation of the phenotypic traits of plants in two field assays

Forty $F_{3:5}$ offspring were selected in the previous assay. Using these offspring and the four parent plants, two field assays were carried out according to a randomized block design with four repeats. One assay was conducted in Embrapa Meio-Norte Experimental Unit, municipality of Teresina, state of Piauí. Seeds were grown with conventional spray irrigation. The second assay was carried out without irrigation in a private company (Agropecuária Milênio) in Tracuateua, state of Pará, Brazil. Quadrats were defined as 0.50-m-wide rows standing 0.70 m apart. The space between plants was 0.25 m. Three seeds were planted in each hole. Lopping was performed and one individual remained in each hole. The following characters were evaluated: (i) time to flowering in days (TF) based on the first blooming in a quadrat; (ii) time in days to maturity (MAT) of the first individual to mature in a quadrat; (iii) pod length (PL) calculated as the mean length of five pods in a quadrat; (iv) number of seeds per pod (NSP) calculated as the mean number of seeds in five pods in a quadrat; (v) weight of 100 seeds (W100S); (vi) seed index (SI) defined as the ratio of the weight of the seeds contained in five pods to the total weight of the five pods considered; (vii) weight of seeds obtained in a quadrat (PROD), (viii) seed length (SL); (ix) seed width (SW); (x) seed height (SH); (xi) seed length-to height ratio

(SLSH), (xii) seed width-to-height ratio (SWSL); (xiii) hilum length (HL); and (xiv) hilum width (HW). All parameters describing seeds were calculated as the mean values for three seeds. The index J was used to describe shape, according to the length-to-height ratio of seeds. The index H defines the seed filling based on the width-to-height ratio of seeds (Puerta Romero, 1961). The statistical software SAS (SAS Institute, 2000) was used to calculate variance and covariance. The Scott-Knott test at 5% probability was used to compare and group means (Zimmermann, 2004) in the software Genes (Cruz, 2007).

Conclusions

All in all, in the present study 14 large seed offspring were obtained (that is, with W100S between 25 and 30 g). In addition, length and width of hilum of offspring were between the values exhibited by small- and large-seed parent plants. Four offspring produced seed with high commercial value, associated with double resistance to CPSMV (serotype I), and CABMV. This is the first time that production of large, filled, white seed with rugose coat and upright plants, with high commercial value and resistant to CABMV and CPSMV is described in Brazil, which represents an important move in the genetic improvement of this culture. The results of the present study will become an important tool in the maintenance and progress of cowpea culture in the country and elsewhere.

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