New hybrids from peanut (*Arachis hypogaea* L.) and synthetic amphidiploid crosses show promise in increasing pest and disease tolerance

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Genet. Mol. Res. 14 (4): 16694-16703 (2015)
Received June 26, 2015
Accepted October 16, 2015
Published December 11, 2015
DOI http://dx.doi.org/10.4238/2015.December.11.17

ABSTRACT. The primary gene pool of the cultivated peanut (*Arachis hypogaea* L., allotetraploid AABB) is very narrow for some important characteristics, such as resistance to pests and diseases. However, the *Arachis* wild diploid species, particularly those from the section *Arachis,* still have these characteristics. To improve peanut crops, genes from the wild species can be introgressed by backcrossing the hybrids with *A. hypogaea.* When diploid species whose genomes are similar to those of the cultivated peanut are crossed, sterile hybrids result. Artificially doubling the number of chromosomes of these hybrids results in fertile synthetic polyploids. The objectives of this study were: 1) to obtain progenies by crossing amphidiploids with the cultivated peanut, and 2) to characterize these two groups of materials (amphidiploids and progenies) so that they

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may be efficiently conserved and used. Using morphological, molecular, and pollen viability descriptors we evaluated one cultivar of *A. hypogaea* (IAC 503), eight synthetic amphidiploids, and the progenies resulting from four distinct combinations of crossing between IAC 503 and four amphidiploids.

Key words: Interspecific hybrid; Germplasm; Characterization; Pollen viability; Groundnut; Wild species

INTRODUCTION

Arachis hypogaea L., the cultivated peanut, is directly consumed and used to produce edible oil, ranking fifth in vegetable oil production in the world (FAOSTAT, 2010). The cultivated groundnut is an allotetraploid (genome formula AABB). This species originated from a cross between two genome diploid species (one A species and one B species), followed by a spontaneous duplication of chromosomes. Molecular (Kochert et al., 1991, 1996; Krapovickas and Gregory, 1994), cytogenetic (Seijo et al., 2004), and crossability and hybrid fertility (Fávero et al., 2006) data all suggest that *A. duranensis* Krapov. & W.C. Gregory donates the A genome and *A. ipaënsis* Krapov. & W.C. Gregory donates the B genome to the cultivated peanut (both belong to the section *Arachis*, one of the nine sections of the genus *Arachis* (Krapovickas and Gregory, 1994)).

Section *Arachis* comprises two allotetraploid (*A. hypogaea* and *A. monticola* Krapov. & Rigoni) and 29 diploid species (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). The genomes of section *Arachis* species were classified for many years as A, B, and D. Recently, analyses of heterochromatic bands and 45S rDNA loci patterns led the species previously classified as B genomes to be arranged into three groups: B, comprising *A. ipaënsis*, *A. magna* Krapov., W.C. Gregory & C.E. Simpson, *A. gregoryi* C.E. Simpson, Krapov. & Valls, *A. valida* Krapov. & W.C. Gregory, and *A. williamsii* Krapov. & W.C. Gregory; K, comprising *A. batizocoi* Krapov. & W.C. Gregory, *A. cruziana* Krapov., W.C. Gregory & C.E. Simpson, and *A. krapovickasii* C.E. Simpson, D.E. Williams, Valls & I.G. Vargas; and F, comprising *A. benensis* Krapov., W.C. Gregory & C.E. Simpson and *A. trinitensis* Krapov. & W.C. Gregory (Robledo and Seijo, 2010). This new classification is supported by systematic molecular studies on the genus *Arachis* (Bechara et al., 2010; Friend et al., 2010; Wang et al., 2010).

Species of section *Arachis* are highly resistant to many pests and diseases that affect *A. hypogaea* (Company et al., 1982; Gardner and Stalker, 1983; Subrahmanyam et al., 1983; Pande and Rao, 2001; Fávero et al., 2009). Because section *Arachis* comprises species that have genomes similar to the A or B genomes of the cultivated peanut, the genes involved in the expression of resistance in the wild species can be introgressed into the cultivated peanut by hybridization. Gene introgression can be conducted by crossing the cultivated species with a diploid species, which results in sterile triploid plants. These hybrids are treated with colchicine to duplicate their chromosomes, and allowed to self-pollinate until they reach the tetraploid level, when they develop four sets of chromosomes. In their analysis of a population that Smart and Gregory (1967) obtained from a cross between *A. cardenasii* Krapov. & W.C. Gregory and *A. hypogaea*, Garcia et al. (1995) reported that gene introgression happened through recombination and chromosome substitution. Alternatively, introgression can be performed by obtaining synthetic allopolyploids from crosses between A and B genome species, then duplicating the chromosomes of the hybrids using colchicine (Simpson, 1991). In contrast to the first approach, the latter allows introgression in both genomes at the same time. Consequently, some research groups are developing interspecific

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hybrids of *Arachis* to introgress wild species genes into *A. hypogaea* (Burow et al., 2001; Fávero et al., 2006; Kumari et al., 2014).

It is important to characterize synthetic hybrids so they can be effectively used and managed. For example, this characterization allows the hybrids themselves to be identified, and therefore distinguished from autogamous plants, which is an essential step in *Arachis* because most of its species are autogamous. It can also help prevent misclassification and the loss of genetic integrity during long-term conservation. Finally, it allows the identification of hybrids with several interesting traits to be used in the subsequent steps of the introgression program.

Different types of molecular markers can be used to identify hybrids. In this study, we used microsatellites because they are codominant, multi-allelic, polymorphic, and transferable markers. The objectives of this study were to obtain progenies resulting from crossings between amphidiploids resistant to leaf diseases (late and early leaf spot and rust) and the cultivated peanut, and to characterize these two groups of materials for their efficient conservation and use.

MATERIAL AND METHODS

Material

The morphological characterization was performed in seven synthetic amphidiploids (An 4, An 7, An 8, An 9, An 10, An 11, and Di 07T) (Table 1). Four synthetic amphidiploids (An 9, An 10, An 11, and Di 07T) and the cultivar IAC 503 from *A. hypogaea* were also used in hybridizations, and their progenies were characterized using microsatellite markers so hybrids could be identified. After hybrids were identified, progenies were characterized morphologically and for pollen viability.

Table 1. Characterized genotypes.

Code	Genotype	Genome*
cv. IAC 503	*A. hypogaea*	AABB
An 4	(*A. ipaënsis* K30076 x *A. duranensis V* 14167)^{4x}	BBAA
An 7	(*A. vallsii* Krapov. & W.C. Gregory V 7635 x *A. williamsii* Wi1118)^{4x}	nnBB
An 8	(*A. magna V* 13751 x *A. cardenasii* GKP 10017)^{4x}	BBAA
An 9	(*A. gregoryi V* 6389 x *A. stenosperma* Krapov. & W. C. Gregory V 12488)^{4x}	BBAA
An 10	(*A. magna* KG 30097 x *A. stenosperma V* 15076)^{4x}	BBAA
An 11	(*A. vallsii V* 7635 x *A. stenosperma V* 10229)^{4x}	nnAA
Di 07T	(*A. batizocoi* K 9484 x *A. helodes* Martius ex Krapov & Rigoni V6325)^{4x}	KKAA

*nn indicates not yet identified.

Methods

Hybridization

Hybrids were created using amphidiploids as male parents and the *Arachis hypogaea* L. cultivar IAC 503 as the female parent (Table 2). The hybridization consisted of emasculating the flowers of the female parent during the bud phase. The following morning, emasculated flowers were pollinated with fresh pollen from the male parent.

Table 2. Hybridization outcomes.

Cross (male x female)	No. of pollinations	No. of hybrids identified	Percentage of success	No. of F2 seeds
IAC503 x An 9	115	2	1.74	0
IAC503 x An 10	129	12	9.30	437
IAC503 x An 11	65	11	16.92	34
IAC503 x Di 07T	86	38	44.19	10
Total	395	63/138		

Molecular characterization

A total of 32 microsatellite loci were screened for polymorphisms using the five parents, which included the four amphidiploids and the *A. hypogaea* cultivar IAC 503. Each reaction contained 3 µL DNA (2.5 ng/µL), 4 µL primer pairs (1 µM), 1.3 µL 10X polymerase chain reaction (PCR) buffer, 0.6 µL $MgCl_2$, 1.3 µL bovine serum albumin (2.5 mg/mL), 1.3 µL dNTPs (2.5 mM), 0.25 µL *Taq* DNA polymerase (5U/µL), and 1.25 µL water. The cycling conditions were as follows: 5 min at 94°C, followed by 30 1-min denaturation cycles at 94°C, 1 min of annealing at 56-60°C, 1 min of extension at 72°C, and a final 7-min extension at 72°C. Table 3 lists the annealing temperatures of primer pairs for which polymorphisms were detected in at least one pair of parents. The PCR products were electrophoresed on 1.5% agarose gel at 120 V for 2 h and stained with ethidium bromide, and the fragments were viewed under ultraviolet light.

Table 3. Microsatellite polymorphic loci between crossed parents.

Locus	Primer (5'-3')	Annealing temperature (ºC)	Polymorphic progenitor
seq14E10	TTGACAAAATAACCTCACTT ACCTAGTGGGACAAGGCTTT	56	IAC503 x An9
seq13A7	AACTCGCTTGTACCGGCTAA AATCCGACGCAATGATAAAA	58	IAC503 x An10, IAC503 x An 11
seq15D2	TTTTGGCTACCTTTGCTGCT TATATTGTCCTGCAAGCCCG	60	IAC503 x An 9
seq12E3	CTCACACCAATCAGTCGACA GACCAAAACAAAATTTGGAA	56	IAC503 x An9, IAC503 x An10
seq10C12	TTCACTTCCTAACCGAGCTC CAAACTTGGGCCAACAGACT	58	IAC503 x An10, IAC503 x An11, IAC503 x Di07T,
seq13E6A	GTCACGTAATTGGATGCACG TGGCAATTTATTGATGCAGG	58	IAC503 x An9, IAC503 x An10

Morphological characterization

The morphological characterization was conducted at Embrapa Genetic Resources and Biotechnology, Brasilia, Distrito Federal, Brazil, using leaf, flower, and plant structure descriptors. We collected four samples of expanded leaves (counting from the apex of the branch), as well as four flower samples from each hybrid plant and progenitors. We used descriptors established in the literature (IBPGR, 1990; Monçato, 1995), with minor modifications.

Descriptors of the main axis and lateral branches included basal and distal leaflet length and width, petiole and petiolule length, length of the free and adnate parts of stipules, stipule width, the presence of trichomes and bristles on the petiole and petiolule, trichomes on the abaxial and adaxial faces of the leaflets (border, center, and midvein), trichomes and bristles on the free and adnate part of stipules (border and center), anthocyanin on the stipule, bristles on the border of the leaflet, and bristles on the free and adnate parts of stipules. The flower descriptors were standard

length and width, wing length and width, hypanthium length, lower and upper lip length, and flower color. Plant height and the lengths of lateral branches were also measured.

Morphological differences between the 64 descriptors of hybrids and progenitors were analyzed using principal components analysis and Pearson's correlations with SAS software, version 8.2, using PROC COMP and Microsoft Excel.

Pollen viability

The fertility of interspecific hybrids of *Arachis* was estimated using pollen staining. Between 8:00 and 9:30 am, four flowers of each hybrid were randomly chosen and collected. Pollen was distributed on slides with a drop of 2% acetocarmine with glycerin, after which a coverslip was placed on the sample and slight pressure was applied. An optical microscope (Olympus model BH-2) was used to count, in random order, four replications of 100 pollen grains (including viable and non-viable grains) for each sample. Only two hybrids (Di07T and IAC 503 x An 9) were not evaluated because they produced too few flowers. An analysis of variance compared the average fertility of each hybrid and progenitor. Tukey's tests were then used to identify differences between each hybrid and progenitor.

RESULTS AND DISCUSSION

Hybridization and hybrid identification

Four different combinations of IAC 503 and four different amphidiploids (An 9, An 10, An, 11, and Di 07T) were studied, for a total of 395 hybridizations (Table 2). Sixty-three progenies were identified as hybrids using molecular markers. Table 2 shows the combinations of progenitors used, the number of pollinations obtained, the number of hybrids identified using single-sequence repeats, the percentage of success (ratio of the number of hybrids to the number of pollinations), and the number of F_2 seeds. The most prolific hybrid developed was IAC 503 x An 10; although its percentage of success was 9.3%, it produced 437 F_2 seeds. The microsatellite primer pairs used were developed for *A. hypogaea* (Ferguson et al., 2004). The transferability of microsatellite primers was high, as observed in many other studies on *Arachis* species (Bravo et al., 2006; Hoshino et al., 2006; Moretzsohn et al., 2009; Koppolu et al., 2010).

The polymorphic loci for at least one pair of parents are listed in Table 3. Polymorphisms between *A. hypogaea* (first three lanes) and several other parents can be seen in Figure 1.

The pair of parents with the largest number of polymorphic loci (five) was IAC 503 and An 8 (*A. magna* x *A. cardenasii*)^{4x}, and the pair with the least (one polymorphic locus) was IAC 503 and Di07T (*A. batizocoi* x *A. helodes*)^{4x}. Several authors have shown *A. magna* to be very similar to *A. ipaënsis* (Krapovickas and Gregory, 1994; Milla et al., 2005; Moretzsohn et al., 2009), which is the donor of the B genome to the cultivated peanut (Kochert et al., 1996). *A. batizocoi* was for many years considered a B genome, but was recently re-classified as a K genome species (Robledo and Seijo, 2010). Based on phylogenetic data, *A. batizocoi* is more distantly related to *A. hypogaea* than *A. magna,* while *A. cardenasii* and *A. helodes*, both A genome species, form a monophyletic group (Bechara et al., 2010). Therefore, we expected to observe more polymorphisms between IAC 503 and Di 07T than between IAC 503 and An 8. Our findings of more polymorphisms between IAC 503 and An 8 may be due to high variability in the microsatellite loci.

The variability in the number of hybrids resulting from each cross we evaluated could have several causes, with the most probable being pre-fertilization or post-fertilization barriers. There is

evidence of pre- and post-zygotic barriers between *Arachis* species (Tallury et al., 1995); however, to date these barriers have been found in wild diploid species rather than in synthetic polyploids, such as the ones used here.

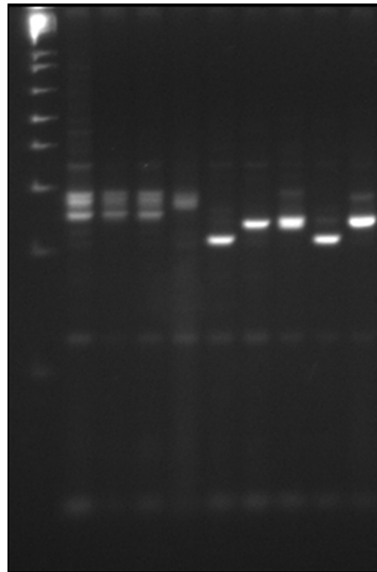

Figure 1. Polymorphisms among *Arachis hypogaea* (first three lanes) and An 6, An 7, An 8, An 9, An 10, and An 11.

Morphological characterization

Of the 64 evaluated descriptors, 13 exhibited no morphological variation among hybrids (on either the main axis or lateral branches: trichomes on the border of the abaxial and adaxial sides of the leaflets, trichomes in the center and midvein of the adaxial side of the leaflets, trichomes on the petiole; on the main axis only: trichomes on the petiolule and trichomes on the free part of the stipule, center, and border). Eigen values indicated that the two first components explained 84.76% of the total variance in morphological characteristics. Nine characteristics emerged as important in genotype discrimination, based on the results of the principal component analysis. The order of contribution to the discrimination of genotypes (and respective values of eigenvectors) was as follows: distal leaflet length on lateral branches (cfdRL: 0,8273); distal leaflet length on the main axis (cfdEC: 0,3311); basal leaflet length on the main axis (cfpEC: 0,3090); distal leaflet width on the main axis (lfdEC: 0,1349); petiolule length on lateral branches (cpoRL: 0,1207); distal leaflet width on lateral branches (lfdRL: 0,1168); free part of stipule width on lateral branches (lelRL: -0,1158); free part of stipule length on lateral branches (celRL: 0,1070); and basal leaflet width on the main axis (lfpEC: 0,1047). These results should facilitate future investigations of these genotypes.

Also using principal component analysis, Fávero (2004) used 15 descriptors for the genotype discrimination of wild *Arachis* species. Six of those were used in the present study (basal and distal leaflet length and width on the main axis, distal leaflet length and width on lateral branches). Pearson's correlation coefficients were calculated for the nine main descriptors (Table 4). These correlations may be due to functional and/or structural proximity between the structures.

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Table 4. Pearson correlation coefficients among the nine main morphological characteristics measured.

	cfpEC	lfpEC	cfdEC	lfdEC	cfdRL	lfdRL	cpoRL	celRL
lfpEC	0.6540							
cfdEC	0.9827	0.7179						
lfdEC	0.6082	0.9795	0.6894					
cfdRL	0.6422	0.5481	0.6897	0.5360				
lfdRL	0.8164	0.3596	0.7961	0.3024	0.4049			
cpoRL	0.7829	0.3842	0.7509	0.2890	0.3619	0.9713		
celRL	0.4806	0.7600	0.5470	0.7432	0.4821	0.3060	0.3665	
lelRL	-0.2471	-0.4237	-0.2978	-0.5135	-0.4737	0.1772	0.2909	-0.0451

cfpEC, basal leaflet length on the main axis; lfpEC, basal leaflet width on the main axis; cfdEC, distal leaflet length on the main axis; lfdEC, distal leaflet width on the main axis; cfdRL, distal leaflet length on the lateral branch; lfdRL, distal leaflet width on the lateral branch; cpoRL, petiolule length on the lateral branch; celRL, free part of stipule length on the lateral branch; lelRL, free part of stipule width on the lateral branch. Italicized values indicate significant correlations at $P < 0.05$.

A biplot graph was obtained (Figure 2) by multiplying the values of the main characteristics observed in Components 1 and 2 by the average values of each characteristic for each genotype. The hybrid IAC 503 x An10 was closer to the progenitor An10 than to the progenitor IAC 503, and the hybrid IAC 503 x An11 was closer to An11 than to IAC 503. The amphidiploids An7, An8, An9, An10, and An11 exhibited many morphological similarities. Consequently, all of the hybrids were closer to the wild progenitors than to *A. hypogaea cv* IAC 503, except for hybrid IAC 503 x An9, which was morphologically more similar to IAC 503.

Table 5 shows the average sizes of the nine main characteristics measured. These data are congruent with each plant's growth habit; for example, all of the hybrids were prostrate, as were the wild progenitors, whereas the hybrid IAC 503 x An 9 had a bunching habit, similar to some varieties of *A. hypogaea*. Data for the progenitor Di 07T were not evaluated because the plant was not in good enough condition for morphological characterization and pollen viability observations. However, we expect results for this hybrid to be similar to those of the other amphidiploids.

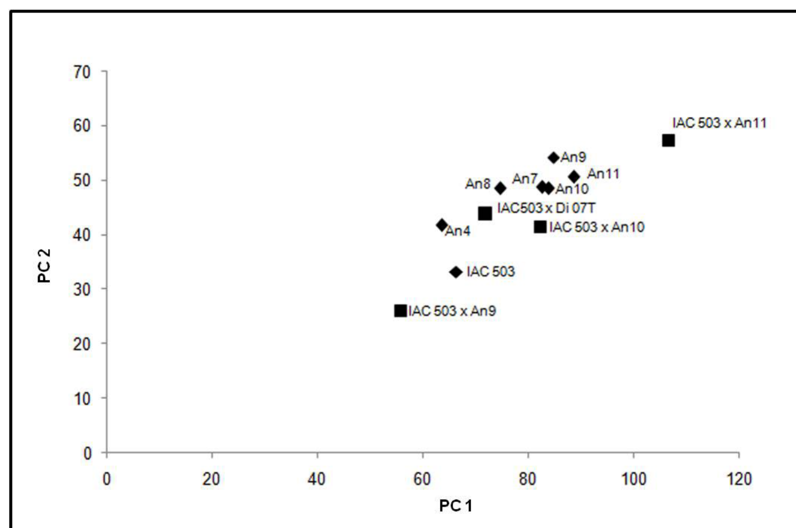

Figure 2. Biplot graph based on a principal component analysis of the nine main descriptors for PC 1 (x-axis) and PC 2 (y-axis). Diamonds indicate the progenitors and squares indicate the interspecific hybrids.

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Table 5. Average size (mm) of the nine main characteristics for each evaluated genotype of *Arachis*.

Genotype	cfpEC	lfpEC	cfdEC	lfdEC	cfdRL	lfdRL	cpoRL	celRL	lelRL
IAC 503	38.23	15.24	43.87	18.53	36.67	19.02	11.11	21.28	3.15
IAC 503 x An 9	28.22	13.30	36.38	16.63	32.22	17.03	9.53	18.35	2.73
An 9	57.30	26.72	67.65	34.57	38.46	26.80	9.72	14.16	4.84
IAC 503	38.23	15.24	43.87	18.53	36.67	19.02	11.11	21.28	3.15
IAC 503 x An 10	47.35	19.88	53.53	23.67	45.91	26.53	13.37	22.78	3.63
An 10	55.95	24.55	61.30	29.74	41.06	22.90	10.14	21.42	4.61
IAC 503	38.23	15.24	43.87	18.53	36.67	19.02	11.11	21.28	3.15
IAC 503 x An 11	68.49	26.85	76.01	32.41	55.64	30.51	11.79	24.35	4.78
An 11	59.46	18.91	68.83	23.68	44.14	23.81	9.01	21.40	4.28
IAC 503	38.23	15.24	43.87	18.53	36.67	19.02	11.11	21.28	3.15
IAC 503 x Di 07T	43.81	19.05	50.66	23.21	35.68	29.83	12.93	20.33	3.16
An 4	40.98	20.64	47.40	25.06	29.14	23.94	10.85	17.54	4.47
An 7	58.45	17.97	62.65	23.77	40.28	21.78	9.04	21.10	4.53
An 8	45.25	26.15	56.49	35.66	34.27	30.76	11.23	14.60	4.71

cfpEC, basal leaflet length on the main axis; lfpEC, basal leaflet width on the main axis; cfdEC, distal leaflet length on the main axis; lfdEC, distal leaflet width on the main axis; cfdRL, distal leaflet length on the lateral branch; lfdRL, distal leaflet width on the lateral branch; cpoRL, petiolule length on the lateral branch; celRL, free part of stipule length on the lateral branch; lelRL, free part of stipule width on the lateral branch.

Pollen viability

The percentages of pollen viability for the cultivar of *A. hypogaea*, the amphidiploids, and of the three F_1 hybrids are listed in Table 6. The IAC 503 x An 9 plants were not evaluated for pollen viability because they did not produce enough flowers. *A. hypogaea* and the amphidiploids exhibited the highest percentages of viable pollen, ranging from 84.00 to 88.67%. Only one hybrid did not differ from the group cited above, IAC 503 x An 10. These data corroborate our findings that F_1 plants produced 437 seeds (shown in Table 2). The other two interspecific hybrids had the lowest percentages of pollen viability (65.50% for IAC 503 x An 11 and 53.75% for IAC 503 x Di 07T). However, they were fertilized and produced a few seeds (Table 2).

Table 6. Average percentage of pollen viability of *Arachis* interspecific hybrids.

Genotype	Pollen viability (%)
IAC 503	88.67[a]
An 7	88.50[a]
An 10	86.00[a]
An 4	84.50[a]
An 8	84.25[a]
An 11	84.25[a]
An 9	84.00[a]
IAC 503 x An 10	71.75[ab]
IAC 503 x An 11	65.50[b]
IAC 503 x Di 07T	53.75[b]

Genotypes with the same superscript letters were not significantly different from each other at $P < 0.05$.

CONCLUSIONS

The cultivated peanut has a narrow gene pool in terms of pest and disease resistance. We obtained synthetic amphidiploids for the purpose of introgression into the cultivated peanut, which may eventually improve its pest and disease resistance. The results demonstrate that it is possible to produce new F_1 interspecific hybrids besides those documented to date. The

morphological characterization of our new hybrids revealed that most were more similar to the amphidiploid progenitors than to the *A. hypogaea* progenitor, except for the IAC 503 x An 9 hybrid. The most prolific combination achieved was IAC 503 x An 10, the amphidiploids produced by the hybridization and polyploidization of *A. magna* KG 30097 and *A. stenosperma* V 15076. This interesting germplasm should continue to be studied in order to develop new breeding lines.

Conflicts of interest

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank Embrapa and CNPq for financial support.

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