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PLANT PROTECTION - Article

Characterization of diploid *Arachis* interspecific hybrids for pest resistance

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ABSTRACT: In Brazil, the thrips (*Enneothrips flavens* Moulton) and rednecked peanutworm (*Stegasta bosquella* Chambers) are considered key pests for the peanut crop. Wild species show resistance to both of these pests, and can be used in breeding programs. The production of the sterile interspecific hybrids is necessary, which could be colchicine treated to get a synthetic amphidiploid with the same or similar genomic configuration of cultivated peanut. In this context, this study proposed the hybridization of wild pest-resistant species in 18 distinct combinations, obtaining the interspecific hybrids of *Arachis* and completing their characterization by (i) the reproductive characterization through pollen stainability with 2% acetocarmine (AC) solution with glycerin and 0.25% tetrazolium solution (TZ), (ii) the molecular certification of hybridization using microsatellite markers, and (iii) the morphological characterization using 61 morphological traits with Principal Component Analysis. Using reproductive, morphological and molecular characterizations, we identified six hybrid plants of the following crosses: three from *A. magna* V 13751 × *A. kuhlmannii* V 9243, two from *A. magna* V 13751 × *A. kempff-mercadoi* V 13250, and one from *A. magna* K 30097 × *A. kuhlmannii* V 7639. Amphidiploids can be reached based on these diploid plants and they can be used in breeding programs aiming pest resistance introgression.

Key words: wild species, pre-breeding, *Enneothrips flavens*, *Stegasta bosquella*.

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INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an oilseed crop and due to its flavor and high oil content, it is used in food industries and has potential for biofuel production (Santos et al. 2005). There are many pests that limit the production of peanut, however, thrips and rednecked peanutworm are currently considered the key pests limiting the production for the crop in the state of São Paulo in Brazil (Michelotto et al. 2013; 2017). Thrips are small sucking insects of the order Thysanoptera, usually measuring between 0.5 and 5.0 mm in length. Most thrips feeds on plant material (phytophagous) and, when feeding, they destroy the leaflets of plants while they are still closed, and can also be vectors for other organisms (Borror and De Long 1969). The rednecked peanutworm (*Stegasta bosquella*) is an insect of the order Lepidoptera, whose adult usually has between 6.0 and 7.0 mm in length. The caterpillar of this insect is phytophagous attacking the leaflet of peanut while they are still closed (Borror and De Long 1969).

One way to control these pests is by obtaining resistant cultivars. However, the resistance located in *A. hypogaea* genotypes is partial. Several wild species of *Arachis* are highly resistant to pests, such as rednecked peanutworm and thrips (Janini 2011[3]). Brazil is the center of origin and holder of the largest genetic diversity of the genus *Arachis* (Freitas et al. 2007), thus the appropriate conservation and characterization of germplasm are of great importance for the use in breeding and biotechnology of peanut.

The main barrier to breeding is that *A. hypogaea* is allotetraploid (4n = 40), while the vast majority of wild species of Section *Arachis* is diploid (2n = 20) (Fernández and Krapovickas 1994). The amphidiploid is an interspecific hybrid having a complete diploid chromosome set from each parent form and this is a viable route for introgression of genes located in wild species of *Arachis*. Thus, the strategy consists of crossing species of two distinct genomes (for example, A × B), obtaining sterile diploid hybrids AB (2n = 20). These hybrids should be treated with colchicine to double the chromosomes and to obtain fertile hybrids with two distinct full genomes, the AABB amphidiploid (4n = 40), which can be crossed with *A. hypogaea* AABB (4n = 40) (Simpson 1991). The same can be carried out using species of genome K in place of genome B.

[3]Janini, J. C. (2011). Resistência de germoplasma silvestre de amendoim (Arachis spp.) a Enneothrips flavens Moulton, 1941 (Thysanoptera:Thripidae) e Stegasta bosquella (Chambers, 1875) (Lepidoptera: Gelechiidae) (PhD Thesis). Jaboticabal: Universidade Estadual Paulista.

In this way, this study aimed to obtain interspecific hybrids of *Arachis* and characterize them by using morphological and reproductive descriptors and to certify the hybridization using molecular markers, with an objective of future introgression of resistance to two key pests in elite genotypes of peanut.

MATERIALS AND METHODS

In this study, we performed two crossings and characterization seasons besides the hybridization certification using molecular markers.

The crossings, reproductive and morphological characterization were performed in plants 90 days after germination.

Germination

Eight seeds from each parent and all progenies seeds were treated with Thiram® and placed on soaked 0.65% Ethrel blotter paper. Germination conditions were 16 h at 20 °C in the dark and 08 h at 35 °C under fluorescent light. Seedlings were transplanted in pots with 180 mg of substrate. When the plants were vigorous they were transplanted to 25 × 40 × 40 cm pots.

Crosses

The crosses were manually performed in a greenhouse, at Embrapa Pecuária Sudeste (São Carlos, state of São Paulo, Brazil) in 2012/2013 and 2013/2014 crossing seasons, from December to March. Flower buds of the female parents were emasculated in the late afternoon and pollinated in the morning of the next day. Eighteen combinations were performed between species of genome B (*A. gregory* C.E. Simpson, Krapov. & Valls VS 14760; *A. magna* Krapov., W.C. Gregory & C.E.Simpson KGSSc 30097 and VSPmSv 13751), K (*A. krapovickasii* C.E. Simpson, D.E. Williams, Valls & I.G. Vargas WiSVg 1291), and genome not yet identified (*A. vallsii* Krapov. & W.C. Greg. VRGeSv 7635) used as female parents, and species of genome A (*A. helodes* Mart. ex Krapov & Rigoni CoSzSv 6862, VSGr 6325; *A. kempff-mercadoi* Krapov., W.C. Greg. & C.E.Simpson V 13250; *A. kuhlmannii* Krapov. & W.C. Greg. VSGr 6413, VRGeSv 7639 and VPoBi 9243) as male parents (Table 1). →

Table 1. Accessions used as parents resistant to pest, species, genome, individual, collectors, latitude, longitude and altitude of the collection area.

Collector code	Species	Genome[1]	Lat. (W)	Long. (S)	Alt. (M)
V 14760	*A. gregoryi* C.E.Simpson, Krapov. & Valls	B	15°29'	60°13'	245
Co 6862	*A. helodes* Martius ex Krapov & Rigoni	A	15°22'	56°13'	175
V 6325	*A. helodes* Martius ex Krapov & Rigoni	A	15°52	56°04'	150
V 13250	*A. kempff- mercadoi* Krapov., W.C. Gregory & C. E. Simpson	A	17°45'	63°10'	280
Wi 1291	*A. krapovickasii* C.E.Simpson, D.E. Williams, Valls & I.G. Vargas	K	18°14'	60°51'	317
V 9243	*A. kuhlmannii* Krapov. & W. C. Gregory	A	18°52'	56°16'	100
V 7639	*A. kuhlmannii* Krapov. & W. C. Gregory	A	20°15'	56°23'	125
V 6413	*A. kuhlmannii* Krapov. & W. C. Gregory	A	15°47'	57°25'	200
K 30097	*A. magna* Krapov., W.C. Gregory & C. E. Simpson	B	16°22'	60°04'	380
V 13751	*A. magna* Krapov., W.C. Gregory & C. E. Simpson	B	16°16'	59°27'	530
V 7635	*A. vallsii* Krapov. & W. C. Gregory	-	20°05'	56°42'	150

[1]The missing data of the V 7635 genome means that its genome was not yet identified.

The success of hybridization percentage (SP) was calculated by the formula: SP = (number of hybrids/number of pollinations) × 100.

Although the combination *A. magna* (V 13751) × *A. kempff-mercadoi* (V 13250) was previously hybridized (Paula et al. 2017), the crossings were performed again aiming at the characterization and comparison to other diploid hybrids.

Reproductive characterization

Four flowers of each genotype were randomly collected. With the aid of tweezers, pollen was taken from the anthers, placed on slides and stained with 2% acetocarmine solution with glycerin (Stalker et al. 1991; Wondraceck-Lüdke et al. 2015) or 0.25% tetrazolium (Beyhan and Serdar 2008). The anthers that did not shed pollen were put on the slides with a drop of dying solution and macerated. Pollen was analyzed as stained or non-stained under the optical microscope. The criteria to consider pollen as stained and possibly viable were the good development and staining of the grains. Two hundred grains were counted from each flower, totaling 800 grains per individual. The stained pollen percentage was calculated for each sample from all evaluated plants. Analyses of variance and Tukey's test for means comparison were performed using the software Statistical Analysis System (SAS® 9.3) (SAS Institute 2012).

Molecular certification of the hybrids

Total genomic deoxyribonucleic acid (DNA) was isolated from young leaves using the protocol based on Cetyl Trimethyl Ammonium Bromide (CTAB) described by Grattapaglia and Sederoff (1994), with the inclusion of an additional precipitation with 1.2 M NaCl, immediately after CTAB buffer. Quantification of total DNA was performed with a spectrophotometer (NanoDrop ND-1000) and DNA integrity was checked on 1% agarose gel stained with ethidium bromide.

Ten simple microsatellite markers (Table 2) were selected from the literature (Moretzsohn et al. 2013) according to the species included in the present study. The loci were amplified by polymerase chain reaction (PCR). The conditions of markers amplification were established with a temperature gradient PCR to determine the primers annealing temperature in the PCR. After setting specific temperatures for each locus, PCR reactions were performed in a thermocycler BioRad

Table 2. Microsatellite markers used in this study selected from Moretzsohn et al. (2013).

SSR	Amplification Temperature[1] (°C)	Size [2] (bp)
Ah3	50	202
gi-623	52	178
IPAHM-406	59	350
PM3	NA	168
PM36	50	200
seq3D9	58	292
RI2A06	52	159
RM14B11	52	312
RN12E01	52	138
Seq18G9	NA	225

[1]NA = markers showing no suitable amplification in the samples; [2]bp = base pairs.

T100, with a final volume of 15 µl, as follows: 120 ng of genomic DNA, 1U Taq DNA polymerase, 1 × PCR buffer (200 mM Tris pH 8.4, 500mM KCl), 1.5 mM $MgCl_2$, 0.2 µM deoxyribonucleotide triphosphate (dNTP), and 0.165 µM of each primer. The protocol used for amplification consisted of 95 °C for 5 min, 30 cycles (94 °C for 45 s; X °C for 45 s; 72 °C for 45 s), and 72 °C for 10 min, where X °C is the specific annealing temperature of the primers. The amplification was verified on 2.5% agarose gel stained with ethidium bromide.

Markers that succeeded in amplification were subjected to electrophoresis in 6% polyacrylamide gels and stained with silver nitrate (Creste et al. 2001) for fragments visualization. The size of these fragments was estimated by using a 10 bp ladder (Invitrogen). From all SSR tested, we selected the markers that better amplified in the samples evaluated and that presented polymorphism for the parents used in crosses. The progenies that had an allele from the male parent not common to the female parent were considered hybrids.

Morphological characterization

Four leaves of the lateral branch, one leaf of the main axis and four flowers of all individuals were randomly collected. The first totally expanded leaves from the lateral branches and main axis were used to perform the characterization. Sixty-one morphological traits were analyzed (Fávero et al. 2015a; 2015b). According to trait, the sample was measured by using a ruler or a caliper and was observed under a stereomicroscope.

The following traits were evaluated on the main axis and lateral branch: proximal leaflet length and width, distal leaflet length and width, petiole and petiolule length, lenght and width of stipule free part of stipule, lenght of stipule adnate part, trichomes on the abaxial and adaxial leaflet border, trichomes on the abaxial and adaxial leaflet center and midvein, bristles on the leaflet border, trichomes on the petiole and on the petiolule, Bristles on the petiole and on the petiolule, trichomes on the stipule (free and adnate part) center or border, bristles on the stipule (free and adnate part), anthocyanin in the stipule. The flower traits were: standard length and width, wing length and width, lower and upper lip length and hypanthium length.

Data were analyzed using Principal Component Analysis (PCA) in SAS® 9.3 software (SAS Institute 2012). The results of components 1 and 2 were multiplied by the mean values of each trait for each individual and the resulting values were used to build a biplot graph using the software Microsoft Excel®.

RESULTS AND DISCUSSION

Hybridizations

During the hybridization season 3,140 pollinations were performed (Table 3), from which 166 pegs were generated and 49 F_1 seeds were produced (119 pegs produced no seeds). The combination *A. magna* K 30097 × *A. kuhlmannii* V 7639 received the highest number of pollinations (424) but produced only six pegs and four seeds. The accession *A. vallsii* V 7635 crossed with four distinct species showed the greatest difficulty in producing hybrids: 936 pollinations resulted in 11 pegs and five seeds. After all characterizations and certifications performed in this study, six plants were identified as a result of hybridization and the percentage of success was 0.19%.

The accession *A. magna* K 30097, in addition to the resistance to pests (Michelotto et al. 2017; Janini 2011), also presents disease resistance (Fávero et al. 2009), being a material of great importance for the peanut breeding program. Fávero et al. (2015a) performed crosses with four combinations involving *A. magna* K 30097 (*A. simpsonii* V 13710, *A. kuhlmannii* V 10506, *A. kempff-mercadoi* V 13250 and *A. diogoi* K 10602) as female parent and had difficulties to obtain seeds from crosses involving these accessions. Only one of these combinations was used in this study, *A. magna* K 30097 × *A. kempff-mercadoi* V 13250, from which we obtained nine seeds that germinated but died at seedling stage.

Wondraceck-Lüdke et al. (2015) obtained four hybrids from *A. valida* V 13514 × *A. magna* K 30097. But both accessions involved had genome B, unlike combinations with species genome A in this study, indicating that the genetic distance between the materials involved directly affects the production of the hybrid.

All combinations involving accessions *A. krapovickasii* Wi 1291 and *A. vallsii* V 7635 as female parent showed difficulties in obtaining seeds. Three combinations *A. vallsii* V 7635 × *A. helodes* V 6325 and *A. gregory* V 14760 × *A. helodes* Co 6862, *A. kuhlmannii* V 6413 produced no seeds. Several factors can interfere with the hybrids production in *Arachis,* as that the fertilization may not occur after pollination, or be late (pollen tube delayed development), or

Table 3. Combinations between species, genomes, number of pollination (NPO); pegs produced (PP); aborted pegs (AP); seeds (S); alive plants (AlP); dead seedlings (DS); hybrids (H); and percentage of success (PS).

Female parent	-	Male parent	Genome[1]	NPO	PP	AP	S	AlP	DS	H	PS (%)
K 30097 (*A. magna*)	X	V 7639 (*A. kuhlmannii*)	B × A	424	10	6	4	1	3	1	0.24
	X	V 13250 (*A. kempff-mercadoi*)	B × A	113	7	0	9	0	9	0	0.00
	X	V 6325 (*A. helodes*)	B × A	114	11	8	3	1	2	0	0.00
	X	V 9243 (*A. kuhlmannii*)	B × A	116	14	9	5	3	2	0	0.00
V 13751 (*A. magna*)	X	V 7639	B × A	114	6	4	2	0	2	0	0.00
	X	V 13250	B × A	105	7	5	2	2	0	2	1.91
	X	V 9243	B × A	104	22	18	4	3	1	3	2.89
	X	V 6325	B × A	259	20	17	3	0	3	0	0.00
Wi 1291 (*A. krapovickasii*)	X	V 7639	K × A	152	5	2	3	0	3	0	0.00
	X	V 13250	K × A	139	9	7	2	0	2	0	0.00
	X	V 6325	K × A	193	4	0	4	2	2	0	0.00
	X	V 9243	K × A	155	15	12	3	0	3	0	0.00
V 7635 (*A. vallsii*)	X	V 7639	NI × A	234	2	0	2	1	1	0	0.00
	X	V 13250	NI × A	277	4	3	1	0	1	0	0.00
	X	V 6325	NI × A	210	1	1	0	0	0	0	0.00
	X	V 9243	NI × A	215	4	2	2	1	1	0	0.00
V 14760 (*A. gregory*)	X	Co 6862 (*A. helodes*)	B × A	119	12	12	0	0	0	0	0.00
	X	V 6413 (*A. kuhlmannii*)	B × A	97	13	13	0	0	0	0	0.00
				3140	166	119	49	14	35	6	0.19

[1]NI = Genome not yet identified.

the proembryo doesn't grow after the peg reaches the ground or grow very slowly. Other factors that can interfere, as the bad flower manipulation technique in the emasculation and pollination process or hybrid seeds don't germinate or they germinate but without enough vigor to survive in the pots (Nigam et al. 1990; Tallury et al. 1995).

Moreover, three (*A. magna* V 13751 × *A. kuhlmannii* V 9243) and two (*A. magna* V 13751 × *A. kempff-mercadoi* V 13250) hybrid plants had no dormancy and germinated in pots before harvesting. These were transplanted into other pots to develop.

Out of the 49 seeds of the progenies produced from crosses, only nine germinated and survived in pots. Besides these nine seedlings, the five mentioned above were incorporated, which had spontaneous germination in the pots prior to harvesting. Thus, 14 plants were evaluated.

As for the difficulty in obtaining interspecific hybrids in *Arachis*, the number of abortions is a major factor that decreases the percentage of success. In this study, during the crosses, abortions were observed for 117 pegs, which have not developed seeds (Table 3), and during the germination of seeds, two other types of abortions were verified: abortion of well developed seeds that did not germinate, and death of seedlings without enough vigor to survive in the pots. Abortions observed in this study, both pre- and post-zygotic, may indicate that these individuals were hybrids that failed to survive.

The plants resulting from the crossing between *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 were characterized as hybrids. This hybrid was artificially doubled, which generated the amphidiploid An13 (*A. magna* V 13751 × *A. kempff-mercadoi* V 13250)$^{4\times}$. When An13 was crossed with the cultivar IAC OL4, 98 pollinations were undertaken, which resulted in 10 seeds. All these 10 seeds were germinated and four were characterized hybrids. The An13 (AABB) and IAC OL4 (AABB) have similar genomic formulas, so there were fewer abortions of pegs, seeds and seedlings (Paula et al. 2017).

Reproductive Characterization

The analysis totaled 22 plants, eight parents and 14 individuals of the progenies (F_1). For both pollen grain staining methods (Fig. 1), analysis of variance evidenced →

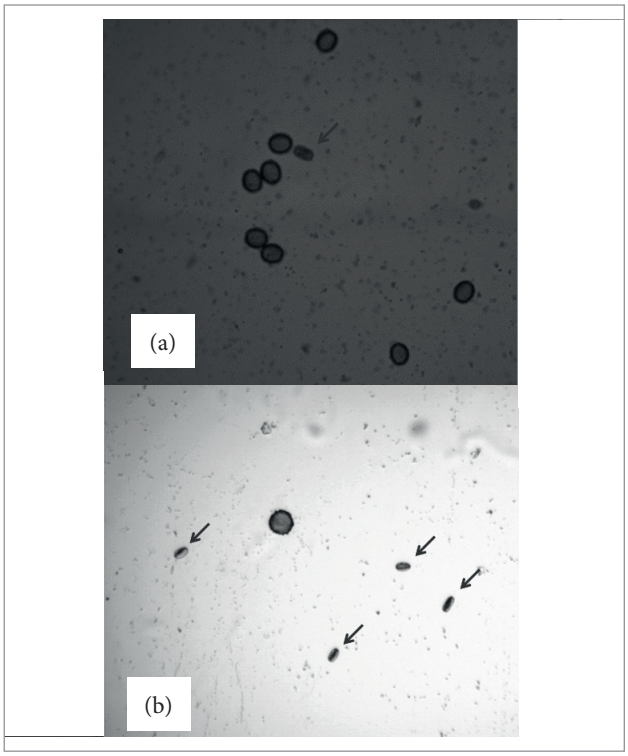

Figure 1. (a) Pollen of the accession V 9243 that was stained with 2% acetocarmine with glycerin. (b) Pollen of the hybrid V 13751 × V 13250 that was stained with 0.25% tetrazolium. Arrows indicate non-stained pollen. 100× magnification in optical microscope.

significant differences between individuals, and no differences between replications.

Regarding the results of CA staining (Table 4), most parents showed high viability (above 96%), except the plant 7 that had lower viability (58%). Out of the 14 plants with potential to be hybrid, eight had high viability (above 86%) and six showed low viability (2.37%). Out of the 22 individuals tested by Tukey's test at 5% probability, the groups *a* to *d* encompassed all parents and individuals from selfing of progenies from crosses *A. krapovickasii* Wi 1291 × *A. helodes* V 6325 (plants 9 and 10), *A. magna* K 30097 × *A. kuhlmannii* V 9243 (pls. 11, 12 and 13), *A. vallsii* V 7635 × *A. kuhlmannii* V 7639 (pl.14), *A. vallsii* V 7635 × *A. kuhlmannii* V 9243 (pl. 15), *A. magna* K 30097 × *A. helodes* V 6325 (pl. 18), whereas the group *e* encompassed the hybrid individuals from the combinations of *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 (pls. 16 and 17), *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19) and *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pls. 20, 21 and 22).

With respect to results of TZ staining (Table 4), most parents showed high viability (above 77% stained grains) except the plant 7 (V 7639), which continued to show viability lower than expected (33%), however, much higher than plants characterized as hybrid. For progenies plants, five of them showed high viability (above 90%) and six showed viability below 1.50%. Tukey's test at 5% probability indicated that the groups *a* to *f* encompassed all parents and individuals from selfing, but the group *g* included the hybrid individuals of progenies *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 (pls. 16 and 17), *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19), *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pls. 20, 21 and 22). Three plants (pl. 11, 12 and 13) were not evaluated because they died.

In the case of reproductive and speciation strategies, Krapovickas and Gregory (1994) stated that selfing is considered the normal mode of reproduction in *Arachis*, but the flower can also receive sporadic visits of insects. In this sense, fertility, as estimated by pollen grain staining, can be used to determine the degree of genetic isolation between species. If more than 50% pollen grains are stained, the anther dehiscence is normal. If less than 50% are stained, anthers begin to have difficulty in dehiscence. Near 25% staining, anthers should not be dehiscent, but the grains may still be captured by a pollinator, such as bees. With less than 15% stained pollen, anthers must be dissected to obtain the pollen grains, and when the rate is below 10%, due to the small number of viable pollen grains, it is doubtful whether it is possible to achieve a successful pollination.

In individuals *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 (pls. 16 and 17), *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19), and *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pls. 20, 21 and 22), anthers were indehiscent and there was difficulty in taking the pollen; anthers had to be macerated on the slide. These F_1 plants obtained from interspecific crosses between accessions of genome A and B were classified as hybrids, and all of them showed a low pollen stainability, indicating high genetic isolation between the parents species. Species with A genome and species with B genome have approximately 20% genetic similarity (Moretzsohn et al. 2013).

Stalker (1991) compared the pollen viability and meiosis in intra and interspecific crosses. All parents presented over 98% of stained grains, a low presence of univalents (below 0.05 univalent) and high rate of bivalents (approximately 10 bivalents) during meiosis, thus, they were considered normal. All progenies individuals resulting from intraspecific crosses showed similar results from the parents, indicating high genetic similarity between the accessions used for →

Table 4. Pollen stainability of parent *Arachis* accessions and progenies.

Individual	Identification	Mean percentage viability of pollen grains according to the staining[1]	
		CA[2]	TZ[3]
A. magna (K 30097) × *A. kuhlmannii* (V 9243)	12	99.50 a	NE[4]
A. magna (K 30097) × *A. helodes* (V 6325)	18	99.12 ab	96.00 ab
A. kuhlmannii (V 9243)	6	99.00 ab	90.00 c
A. magna (K 30097) × *A. kuhlmannii* (V 9243)	11	98.87 ab	[4]NE
A. krapovickasii (Wi 1291) × *A. helodes* (V 6325)	10	98.83 ab	96.33 ab
A. krapovickasii (Wi 1291)	1	98.62 ab	96.66 ab
A. magna (K 30097)	2	98.50 ab	98.37 a
A. vallsii (V 7635) × *A. kuhlmannii* (V 9243)	15	98.25 ab	94.37 b
A. krapovickasii (Wi 1291) × *A. helodes* (V 6325)	9	97.87 ab	97.00 ab
A. kempff-mercadoi (V 13250)	8	97.87 ab	88.87 c
A. vallsii (V 7635)	4	97.87 ab	82.66 d
A. magna (V 13751)	3	96.75 ab	77.33 e
A. helodes (V 6325)	5	96.50 ab	89.75 c
A. vallsii (V 7635) × *A. kuhlmannii* (V 7639)	14	96.16 b	90.66 c
A. magna (K 30097) × *A. kuhlmannii* (V 9243)	13	86.62 c	NE[4]
A. kuhlmannii (V 7639)	7	58.00 d	33.00 f
A. magna (V 13751) × *A. kempff-mercadoi* (V 13250)	17	2.37 e	1.50 g
A. magna (V 13751) × *A. kuhlmannii* (V 9243)	20	2.37 e	1.25 g
A. magna (V 13751) × *A. kuhlmannii* (V 9243)	21	2.37 e	1.33 g
A. magna (V 13751) × *A. kuhlmannii* (V 9243)	22	2.12 e	1.50 g
A. magna (K 30097) × *A. kuhlmannii* (V 7639)	19	1.25 e	1.12 g
A. magna (V 13751) × *A. kempff-mercadoi* (V 13250)	16	1.00 e	0.87 g
CV %		1.67	1.84

[1]Means followed by same letters are significantly equal at 5% probability by Tukey's test; 22% carmine acetic acid with glycerin; 30.25% Tetrazolium solution; [4]NE = Not Evaluated (plants died).

the hybrids production. Individuals of the progenies from interspecific crosses exhibited less than 11% stained pollen with a high number of univalents and low amount of bivalents, indicating low genetic similarity.

Moretzsohn et al. (2013) showed the genetic similarity between distinct *Arachis* species. The studies of Moretzsohn et al. (2013) and Stalker (1991), combined, show that the genetic similarity between the parents can influence the percentage viability of pollen.

Molecular Identification

The SSR loci selected were Seq3D09, IPAHM406, RI2A06 and RM14B11, based on their amplification pattern in the individuals evaluated, which allowed the identification of hybridization in individuals *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 (pls. 16 and 17), *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19) and *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pls. 20, 21 and 22), and selfing in individuals *A. krapovickasii* Wi 1291 × *A. helodes* V 6325 (pls. 9 and 10), *A. magna* K 30097 × *A. kuhlmannii* V 9243 (pls. 11, 12 and 13) , *A. vallsii* V 7635 × *A. kuhlmannii* V 7639 (pl. 14), *A. vallsii* V 7635 × *A. kuhlmannii* V 9243 (pl. 15) and *A. magna* K 30097 × *A. helodes* V 6325 (pl. 18). Amplification profile of informative alleles from the four SSR loci used is described in Table 5 for all markers and in Fig. 2 for RI2A06 marker. As described in other studies (Schuck et al. 2011; Newaskar et al. 2013; Saltonstall et al.

Table 5. Amplification profile of informative alleles from the four SSR loci used for hybridization confirmation.

ID	Individual	Type	SSR marker/allele				Result
			IPAHM406	Seq3D09	RI2A06	RM14B11	
			272 bp	270 bp	160 bp	342bp	
1	*A. krapovickasii* (Wi 1291)	female parents	NE	NE	NE	A	NA
2	*A. magna* (K 30097)	female parents	A	A	A	A	NA
3	*A. magna* (V 13751)	female parents	A	A	A	NE	NA
4	*A. vallsii* (V 7635)	female parents	NE	NE	NE	A	NA
5	*A. helodes* (V 6325)	male parents	P	P	P	P	NA
6	*A. kuhlmannii* (V 9243)	male parents	P	P	P	P	NA
7	*A. kuhlmannii* (V 7639)	male parents	NE	NE	NE	P	NA
8	*A. kempff-mercadoi* (V 13250)	male parents	P	P	P	NE	NA
9	*A. krapovickasii* (Wi 1291) × *A. helodes* (V 6325)	F_1	NE	NE	NE	A	self
10	*A. krapovickasii* (Wi 1291) × *A. helodes* (V 6325)	F_1	NE	NE	NE	A	self
11	*A. magna* (K 30097) × *A. kuhlmannii* (V 9243)	F_1	A	A	A	NE	self
12	*A. magna* (K 30097) × *A. kuhlmannii* (V 9243)	F_1	A	A	A	NE	self
13	*A. magna* (K 30097) × *A. kuhlmannii* (V 9243)	F_1	A	A	A	NE	self
14	*A. vallsii* (V 7635) × *A. kuhlmannii* (V 7639)	F_1	NE	NE	NE	A	self
15	*A. vallsii* (V 7635) × *A. kuhlmannii* (V 9243)	F_1	NE	NE	NE	A	self
16	*A. magna* (V 13751) × *A. kempff-mercadoi* (V 13250)	F_1	P	P	P	NE	hybrid
17	*A. magna* (V 13751) × *A. kempff-mercadoi* (V 13250)	F_1	P	P	P	NE	hybrid
18	*A. magna* (K 30097) × *A. helodes* (V 6325)	F_1	A	A	A	NE	self
19	*A. magna* (K 30097) × *A. kuhlmannii* (V 7639)	F_1	NE	NE	NE	P	hybrid
20	*A. magna* (V 13751) × *A. kuhlmannii* (V 9243)	F_1	P	P	P	NE	hybrid
21	*A. magna* (V 13751) × *A. kuhlmannii* (V 9243)	F_1	P	P	P	NE	hybrid
22	*A. magna* (V 13751) × *A. kuhlmannii* (V 9243)	F_1	P	P	P	NE	hybrid

A = allele absence; P = allele presence; NE = not evaluated in this sample; NA = not applicable.

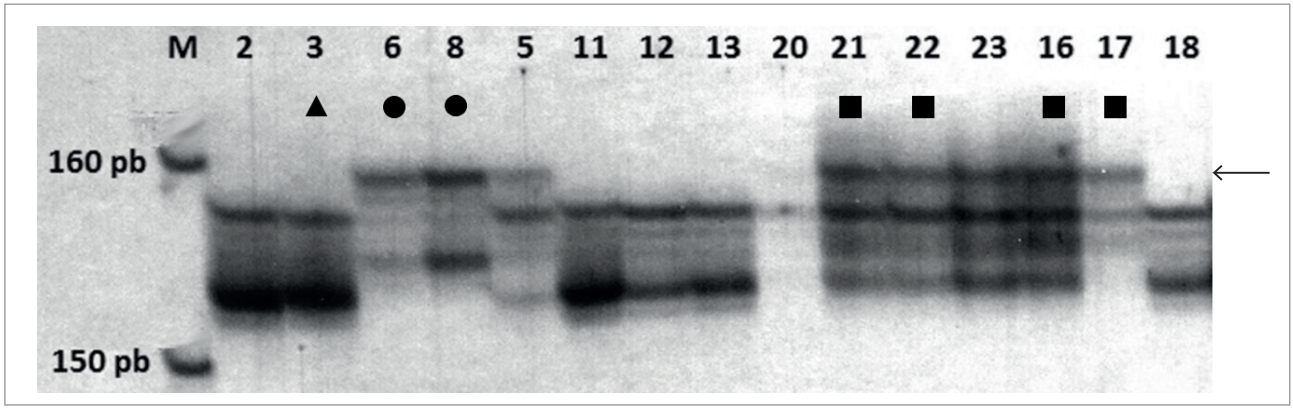

Figure 2. Amplification profile of the marker RI2A06 on 6% polyacrylamide gel for the tested individuals. Individuals: K 30097 (2); V 13751 (3); V 9243 (6); V 13250 (8); V 6325 (5); K 30097 × V 9243 (11); K 30097 × V 9243 (12); K 30097 × V 9243 (13); V 13751 × V 9243 (20, 21 and 22); V 13751 × V 13250 (16 and 17); K 30097 × V 6325 (18). The arrow indicates the polymorphic band identifying hybridization, the triangle indicates the female parent, circles indicate male parents and squares indicate hybrids. M = 10 bp ladder (Invitrogen).

2014), the microsatellite markers were useful in identifying hybridization in natural or controlled crosses.

Morphological Characterization

Individuals were characterized morphologically and PCA eigenvalues revealed that the first three components explained 95% of the total variance in morphological traits.

Table 6 lists the descriptors that explained most of the variation observed according to the Principal Component 1. Among the 15 most important descriptors, seven were collected from the main axis of the plant, four from the lateral branch and four from flower. All other descriptors, not present in this table, were considered irrelevant in this study to determine the morphological variation between individuals.

From the components 1 and 2 multiplied by the mean values of each trait for each individual, it was possible to build a biplot graph (Fig. 3). Plants 11, 21 and 22 had the central axis broken during development. When analyzing the 15 most important descriptors for the characterization, seven of these belonged to the main axis of the plant, so they were very discriminative and plants that did not have it remained completely scattered in the graph.

The parents were well distributed on the plot. Plants *A. magna* K 30097 × *A. kuhlmannii* V 9243 (pls. 11, 12, 13) and *A. magna* K 30097 × *A. helodes* V 6325 (pl. 18), identified as a result of selfing, were located near the female →

Table 6. Order of descriptors that contributed most to the morphological variation observed in the principal component 1 (Prin 1) of the Principal Component Analysis of the parents and hybrids.

Order	Descriptors	Codes[1]	Individuals[2]										
			2	3	6	7	8	16	17	19	20	21	22
1	Length of adnate part of stipule LB	LapsLB	8.215	7.280	7.383	5.575	7.675	8.420	8.610	5.575	9.275	9.135	8.550
2	Distal leaflet length LB	DlILB	31.040	24.430	34.700	19.985	27.840	34.490	36.310	19.985	28.600	30.565	23.550
3	Bristles on the petiolule MA	BpoMA	1.000	2.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
4	Bristles on the petiole MA	BpMA	1.000	2.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
5	Upper lip length FL	UlIFL	5.690	6.570	8.028	6.063	6.745	7.280	5.980	6.063	6.755	6.715	8.290
6	Standard width FL	SwFL	15.330	14.525	17.503	16.005	14.900	17.165	17.160	16.005	17.420	17.025	17.695
7	Width of free part of stipule LB	Wfps LB	2.985	2.950	3.280	2.995	3.150	3.190	2.795	2.995	2.315	2.455	2.590
8	Petiolule length MA	PolMA	41.740	39.260	11.990	9.330	17.630	40.090	27.240	12.310	29.800	NE[3]	NE[3]
9	Width of free part of stipule MA	WfpsMA	2.860	2.500	3.230	2.420	3.290	2.330	2.360	2.690	1.970	NE[3]	NE[3]
10	Bristles on the leaflet border MA	BlmMA	1.000	1.000	3.000	2.000	2.000	2.000	1.000	3.000	2.000	1.000	1.000
11	Distal leaflet width MA	DlwMA	27.270	20.460	23.750	17.730	14.910	13.530	14.340	20.720	12.170	NE[3]	NE[3]
12	Hypanthium length FL	HlFL	63.190	66.655	43.108	45.545	74.625	58.730	69.080	45.545	75.115	88.075	87.225
13	Trichomes on the petiolule MA	TpoMA	3.000	3.000	3.000	2.000	3.000	3.000	3.000	3.000	3.000	1.000	1.000
14	Standard length FL	SlFL	9.570	10.610	12.193	10.515	11.450	11.240	10.670	10.515	11.230	11.210	12.450
15	Distal leaflet length MA	DlMA	56.030	44.230	49.870	30.190	35.670	37.090	31.830	35.560	28.480	NE[3]	NE[3]

[1]Codes ending with MA refer to the main axis, with LB, to the lateral branch and with FL, to flower. 2Individuals: female parents K 30097 (2), V 13751 (3); male parents V 9243 (6), V 7639 (7), V 13250 (8); hybrid V 13751 × V 13250 (16); V 13751 × V 13250 (17); K 30097 × V 7639 (19); V 13751 × V 9243 (20); V 13751 × V 9243 (21); V 13751 × V 9243 (22). [3] NE = Not Evaluated (the main axis broked).

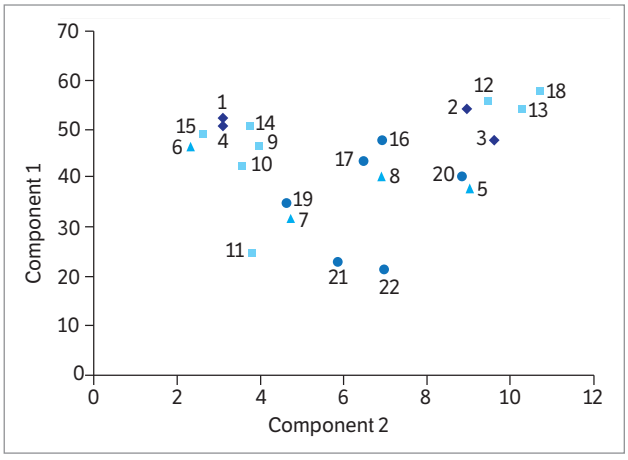

Figure 3. Biplot graph obtained by Principal Component Analysis considering the 61 descriptors for the principal components 1 and 2. Diamonds indicate female parents Wi 1291 (1), K 30097 (2), V 13751 (3), V 7635 (4); triangles, male parents V 6325 (5), V 9243 (6), V 7639 (7), V 13250 (8); and representing the progenies, circles indicate the hybrids V 13751 × V 13250 (16), V 13751 × V 13250 (17), K 30097 × V 7639 (19), V 13751 × V 9243 (20), V 13751 × V 9243 (21), V 13751 × V 9243 (22); and squares, the plants derived from selfing Wi 1291 × V 6325 (9), Wi 1291 × V 6325 (10), K 30097 × V 9243 (11), K 30097 × V 9243 (12), K 30097 × V 9243 (13), V 7635 × V 7639 (14), V 7635 × V 9243 (15), K 30097 × V 6325 (18).

parent *A. magna* K 30097 (pl. 2), in the top right of the graph except *A. magna* K 30097 × *A. kuhlmannii* V 9243 (pl. 11), which did not have the main axis, and stood opposite to these plants, being located in the bottom left of the graph.

Plants of *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pls. 20, 21 and 22), identified as hybrid, were located a little away from their parents. The plant *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pl. 20), the only one that had the main axis, was located closer to the female parent *A. magna* V 13751 (pl. 3) than to the male parent *A. kuhlmannii* V 9243 (pl. 6), the values that caused this distribution in the graph can be seen in Table 6. As well as in *A. magna* K 30097 × *A. kuhlmannii* V 9243 (pl.11), which had no main axis, hybrid plants *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pls. 21 and 22) were located far from the parents.

Hybrids *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 (pls. 16 and 17) were located closer to the male parent *A. kempff-mercadoi* V 13250 (pl. 8) than to the female parent *A. magna* V 13751 (pl. 3). Analyzing the most important descriptor for these two hybrids, the female and male parents had, respectively, 7.67 mm and 7.28 mm for the length of adnate part of stipule in the lateral branch (cpdRL). In this way, plants *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 (pls. 16 and 17), with 8.42 mm and 8.61 mm, respectively, were located closer to the male than to the female parent.

The hybrid *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19) was located closer to the male parent *A. kuhlmannii* V 7639 (pl. 7) than the female parent *A. magna* K 30097 (pl. 2). Considering the most important descriptor, it is observed that the female and male parents presented, respectively, 5.57 mm and 8.21 mm for the length of adnate part of stipule in the lateral branch (LapsLB), and thus the plant *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19), with 5.57 mm, was located closer to the male than to the female parent. This cross presented an important morphological trait, as the female parent *A. magna* K 30097 (pl. 2) has orange flower and the male parent *A. kuhlmannii* V 7639 (pl. 7) has yellow flower. As soon as the first flower appeared in *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19), this had yellow color, as the male parent.

According to Janini (2011), who evaluated 35 accessions of *Arachis*, including wild species, amphidiploid and cultivars, the accessions of *A. kempff-mercadoi* V13250, *A. kuhlmannii* V7639, V 9243, *A. magna* V 13751 and *A. magna* K 30097 are among the accessions that stood out for smaller infestations, greater tolerance and antibiosis to *E. flavens* and *S. bosquella*. Some accessions have multiple defense, protecting against both pests at the same time. In this study, six hybrid plants in three different combinations were produced, two hybrids from *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 (pls. 16 and 17), one from *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19), and three from *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pls. 20, 21 and 22).

The two hybrid plants from *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 (pls. 16 and 17) had more phenotypical traits of the accession *A. kempff-mercadoi* V 13250 (pl. 8), thus making the individual to locate closer to the male parent than to the female parent in the PCA. In agreement with Janini (2011), accession *A. kempff-mercadoi* V 13250 was among those less attacked by *E. flavens* (resistance) and among the least damaged by thrips and rednecked peanutworm (multiple tolerance). According to Michelotto et al. (2017), *A. kempff-mercadoi* V 13250 presented an average of 2.81 thrips per leaf while the controls had averages between 28.99 and 33.64 thrips per leaf. The accession *A. magna* V 13751 was among those with →

lower reductions in plant development (multiple tolerance), among those less attacked by *S. bosquella* (resistance), and among the least damaged by thrips and rednecked peanutworm (multiple tolerance). According to Michelotto et al. (2017), the accession *A. magna* V 13751 presented an average of 6.56 thrips per leaf, while the controls had averages between 28.99 and 33.64 thrips per leaf.

The three hybrid plants from *A. magna* V 13751 × *A. kuhlmannii* V 9243 (pls. 20, 21 and 22) had different characteristics from their parents, this has meant that they were located at a certain distance from the parents in the PCA graph, but closer to the female parent *A. magna* V 13751 (pl. 3) than to the male parent *A. kuhlmannii* V 9243 (pl. 6). According to Janini (2011), the accession *A. kuhlmannii* V 9243 was in the group of the least attacked by *E. flavens* (resistance) and among those with lower reductions in plant development (multiple tolerance).

The hybrid from *A. magna* K 30097 × *A. kuhlmannii* V 7639 (pl. 19) had most of the phenotypic traits more similar to the male parent, and this was located very close to the accession *A. kuhlmannii* V 7639 (pl. 7) in the PCA. According to Janini (2011), the accession *A. kuhlmannii* V 7639 was the one with the best evaluations against *E. flavens* and *S. bosquella*, and among those that suffered minor infestations and damage from both pests (multiple resistance). It was also among those with lower reductions in plant development, damage by trips and rednecked peanutworm and in production, stood out for productivity (multiple tolerance). It affected the development of thrips and rednecked peanutworm in antibiosis test. The accession *A. magna* K 30097 was among those with lower reductions in plant development (multiple tolerance) and among those that affected the development of thrips in antibiosis test. According to Michelotto et al. (2017), *A. magna* K 30097 presented an average of 10.86 thrips per leaf, while the controls had averages between 28.99 and 33.64 thrips per leaf.

They also evaluated three amphidiploids in which some accessions are also present in our study as progenitors. The amphidiploids An8 (V 13751 × GKP 10017)^{4x}, An11 (V 7635 × V 10229)^{4x} and An12 (K 9484 × V 13250)^{4x} had 6.94, 9.85 and 4.33 thrips per leaf respectively, while the controls presented averages between 28.99 and 33.64 thrips per leaf. This study shows that the resistance of wild accessions is maintained even after hybridization and polyploidization.

CONCLUSION

It was possible to get new hybrids derived from interspecific crosses of *Arachis* wild species resistant to pests. Using the reproductive, molecular and morphological identifications, we identified hybridization in six plants of the following crosses: *A. magna* V 13751 × *A. kuhlmannii* V 9243, *A. magna* V 13751 × *A. kempff-mercadoi* V 13250 and *A. magna* K 30097 × *A. kuhlmannii* V 7639, which both parents show resistance to thrips and rednecked peanutworm.

Furthermore, the traits related to the main axis explained most of the morphological variation between individuals, and microsatellite markers RI2A06, IPAHM-406 and RM14B11 were the most informative to identify the hybrids in this study.

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