Transference of multiple resistance to peanut through the development of cross-compatible complex hybrids of wild Arachis

Alessandra Pereira Fávero1*, Adriana Regina Custodio2, Naiana Barbosa Dinato3, Ignácio José de Godoy4, José Guillermo Seijo5 and Marcos Doniseti Michelotto6

1Embrapa Pecuária Sudeste, São Carlos, SP, Brazil.
2Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil.
3Universidade Federal de São Carlos, São Carlos, SP, Brazil.
4Instituto Agronómico, Campinas, SP, Brazil.
5Universidad Nacional del Nordeste, Instituto de Botánica del Nordeste, Facultad de Ciencias Exactas y Naturales y Agrimensura, Corrientes, Argentina.
6Agência Paulista de Tecnologia dos Agronegócios, Polo Centro Norte, Pindorama, SP, Brazil.

Abstract

Peanut (Arachis hypogaea L.) is a tetraploid species with an A and B genome, while the majority of wild Arachis species are diploid with distinct genomes. In pre-breeding programs, one way to introgress interesting wild genes into peanut is by producing amphidiploids. This study aimed at the hybridization between distinct amphidiploids and their characterization, to combine high crossability with peanut, observed in some amphidiploids, with high pest and disease resistances observed in others. These new hybrids were called complex hybrids. Four amphidiploids previously obtained were crossed at four different combinations, and the derived complex hybrids were crossed with four peanut cultivars. Morphological, reproductive, chromosome complement, molecular markers for hybrid identification, phytopatological, and entomological characterizations were performed on the complex hybrids. All cross combinations resulted in complex hybrids. One complete complement of each diploid progenitor was confirmed in each hybrid. Plants of six distinct hybrid combinations were obtained between the complex hybrids and peanut. Based on morphological characterization, differences among progenies from distinct cross combinations were observed. Complex hybrids were considered more resistant to all diseases and pests than peanut cultivars. The simultaneous introgression of genes from four wild Arachis species into peanut was possible through the development of complex hybrids.

Keywords: Amphidiploids, wild species, genetic resources, groundnut, Arachis hypogaea.

Introduction

Peanut (Arachis hypogaea L.) is used worldwide, mainly for oil or grain consumption. The cultivated area is concentrated in tropical and subtropical regions, and the world production in 2016 was estimated at 42.22 million tons (USDA, 2016). Although yield averages above 4.3 Tg ha⁻¹ are recorded under good management practices, the average yield for most of the peanut-producing countries is only 1.28 Tg ha⁻¹ (USDA, 2016). Disease and pest epidemics are leading factors for suppressed yields, and high levels of resistance to many important biotic stresses are not available in the cultivated genepool (Stalker, 2017).

The genetic variability found in wild Arachis species is much higher than that found in cultivated peanuts. The genus Arachis has 82 recognized species and is divided into nine taxonomic sections (Krapovickas and Gregory, 1994; Valls and Simpson, 2005; Valls et al., 2013, Santana and Valls, 2015; Valls and Simpson, 2017). Section Arachis is the most important for peanut breeding, and includes 30 species besides A. hypogaea (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). Wild species of this section are diploid (most are 2n=2x=20 and only two 2n=2x=18) except A. monticola that is tetraploid like the cultivated peanut (Fernández and Krapovickas, 1994; Lavia, 1998; Peñaloza and Valls, 2005). The cultivated peanut and A. monticola are AABB segmental allotetraploids (Fernández and Krapovickas, 1994; Leal-Bertioli et al., 2015). The diploid species were arranged in six different genomes (A, B, D, F, G, and K) according to chromosome morphology, cytogenetic markers, and cross compatibility (Stalker, 1991; Robledo et al., 2009; Robledo and Seijo, 2010; Silvestri et al., 2015).

The difference in ploidy level hinders the direct introgression of genes from wild relatives into the tetraploid pea-
pests and diseases. Many studies show the importance of analyses, and by assays to evaluate resistance to multiple cal, molecular, and cytogenetic markers, by pollen viability here developed were characterized by means of morphologi- cies that are not closely related to peanut (Stalker et al. (Simpson and Starr, 2001).

A. cultivated peanut are then crossed and backcrossed with loids, which can be compatible at different levels with the chromosome number with colchicine. The synthetic amphidip- loids with the high resistance of others in complex hy- brids for the effective introgression of desirable traits into peanut.

The success of introgression of wild alleles, mainly those related to high resistance to pest and diseases, into pea- nut is not only restricted by the ploidy level barrier, but also by the effective recombination within interspecific or inter- genomic hybrids obtained from crosses among more distant species. Different surveys of resistances in Arachis showed that the most interesting performances were detected in species that are not closely related to peanut (Stalker et al., 2016; Stalker, 2017). Moreover, the amphidiploids derived from species that are genetically distant from Arachis hypogaea presented the highest resistances (Michelotto et al., 2016). Therefore, there is a need to combine the high crossability of some am- phidiploids with the high resistance of others in complex hy- brids for the effective introgression of desirable traits into peanut.

In this context, the goal of the present study was to de- velop complex amphidiploids that combine multiple high resistances to diseases and pests and the high crossability with Arachis hypogaea. For that purpose, the complex hybrids here developed were characterized by means of morphologi- cal, molecular, and cytogenetic markers, by pollen viability analyses, and by assays to evaluate resistance to multiple pests and diseases. Many studies show the importance of introgressing Arachis wild genes for pests and diseases resistance, as done in field, laboratory, or greenhouse phenotyping evaluation based on QTL identification (Pande and Rao, 2001; Michelotto et al., 2017; Leal-Bertioli et al., 2015; Zhou et al., 2016). These are the first complex hybrids that include the genome of four distinct species at the same time developed in Arachis, some of them were cross-compatible with peanut, and the derived F₂ showed multiple resistances to pests and fungal diseases.

The results here presented showed that the genomes of four distinct wild species could be used simultaneously for the introgression of alleles into cultivated peanuts. With this approach, it was possible to obtain new complex hybrids and peanut introgressed lines with new interesting allelic combinations for peanut breeding programs.

Material and Methods

Development of complex hybrids

The Arachis species used for obtaining the amphidip- loids are listed in Table 1. All of the Arachis hypogaea accessions used in the introgression crossing are cultivars that are being used by Brazilian producers.

Crosses were performed at Embrapa Recursos Gené- ticos e Biotecnologia, Brazil, from January to May 2005 under greenhouse conditions. Emasculations were carried out in late afternoon and pollination was done in the next early morning.

Four previously obtained synthetic amphidiploids (Fá- vero et al., 2006, 2015) were used in this study (Table 2). Crosses involving four different hybrid combinations were performed: (K 30076 x V 14167)₄ x (K 30006 x V 6325)₄; (K 30076 x V 14167)₄ x (V 6389 x V 9401)₄; (V 6389 x V 9401)₄ x (K 30006 x V 6325)₄; (V 6389 x V 9401)₄ x (K 30006 x G 10017)₄. After harvest, seeds were dried and stored in cold chambers (10 °C/35% RH) until the next growing season.

Table 1 - Accessions of Arachis species, collector code, species name, Brazilian accession code (BRA), municipality, state, or country of collection.

| Accession* | Species                      | BRA  | Municipality | State/Country** | Genome |
|------------|------------------------------|------|--------------|-----------------|--------|
| GKP 10017  | A. cardenasii Krapov. & W. C.Gregory | 013404 | Roboré       | BOL             | AA     |
| VNdEv 14167| A. duranensis Krapov. & W. C.Gregory | 036200 | Salta        | ARG             | AA     |
| VSGr 6389  | A. gregoryi C.E. Simpson, Krapov. & Valls | 012696 | Vila Bela da Ssa. Trindade | MT | BB     |
| VSGr 6325  | A. helodes Martius ex Krapov. & Rigoni | 012505 | S. Antonio do Leverger | MT | AA     |
| KG 30006   | A. hoehnei Krapov. & W. C. Gregory | 036226 | Corumbá      | MS              | K?     |
| cv. IAC Tatu ST | A. hypogaea L.             | 011606 |                | BRA | ABBB   |
| cv. IAC Runner | A. hypogaea L.           | 037389 |                | BRA | ABBB   |
| cv. IAC Caipó    | A. hypogaea L.            | 037371 |                | BRA | ABBB   |
| cv. BR 1      | A. hypogaea L.            | 033383 |                | BRA | ABBB   |
| KGBPscS 30076| A. ipaënsis Krapov. & W. C. Gregory | 036234 | Ipá           | BOL | BB     |
| VP-OBI 9401  | A. lineifolius Valls & C. E. Simpson | 022608 | S. Antonio do Leverger | MT | AA     |

*Collector/Institutional abbreviations: B= Banks; Bi= L.B. Bianchetti; Ev= A. Echeverry; G= W.C. Gregory; Gr= A. Gripp; K= A. Krapovickas; Nv= L. Novara; P= J.R. Pietrarelli; Po= A. Pott; S= C.E. Simpson; Sc= A. Schinini; V= J.F.M. Valls.

**Country or state: ARG= Argentina; BOL= Bolivia; BRA= Brazil; MT= Mato Grosso; MS= Mato Grosso do Sul; SP= São Paulo.
**Development of hybrids between complex hybrids and peanut**

The F1 complex hybrids here obtained were crossed with four cultivars of *A. hypogaea* (*A. hypogaea* subsp. *fastigiata* var. *fastigiata* cv. IAC-Tatu-ST and cv. BR-1, *A. hypogaea* subsp. *hypogaea* cv. IAC-Runner 866 and *A. hypogaea* cv. IAC-Caiapó) (Table 3).

**Morphological characterization of the complex hybrids**

Twenty five morphological characteristics were evaluated in the main axis, in lateral branches, and in the flowers of plants kept under greenhouse conditions. Leaflet descriptors were measured in the first expanded leaves, in four replications of each genotype. The morphological descriptors of the main axis (MA) and lateral branch (LB) were: length and width of the apical and basal leaflets, length of petiole and petiolule, length and width of the stipule fused portion, length of the stipule free portion. The morphological descriptors of the flowers (F) were: length and width of the standard and wing, hypanthium length, length of the posterior and inferior lips. The measurements were taken in millimeters with a digital caliper. Data were analyzed using the analysis of variance and Tukey test and based on Principal Component Analysis.

**Reproductive characterization of complex hybrids**

Pollen viability estimations were performed by staining with 2% glycerol-acetic carmine. Four flowers were collected from each plant and 200 pollen grains were counted per flower. Data were analyzed by analysis of variance and Tukey test.

**Identification of hybrids using SSR markers**

Progenies and parents were analyzed by microsatellite markers (Table 4) developed for *A. hypogaea* (Moretzsohn et al., 2013). Total genomic DNA was extracted from young and fresh leaflets of 41 genotypes, including parents and progenies individuals, according to the method of Doyle and Doyle (1990). The amount and quality of the DNA were evaluated by 1% agarose gel electrophoresis. PCR assays were run with 0.2 µL DNA *Taq* polymerase (5 U/µL), 0.5 µL buffer (with Mg), 1.0 µL dNTPs (2.5 mM), 1.0 µL ultrapure water, 1.2 µL BSA (2.5 mg/mL), 0.1 µL of each primer (10 µm) and 2.0 µL genomic DNA, in a final volume of 6 µL. Amplification reactions were performed in an ABI 9700 (Applied Biosystems, Foster City, CA, USA) thermal cycler, under the following conditions: 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 58 °C for 1 min (depending on annealing temperature of the primer), 72 °C for 1 min, and final extension at 72 °C for 1 min. The allelic detection of 30 SSR loci was performed in an ABI377 automated sequencer in a multiplex loci system (Table 4). Genetic diversity was analyzed by the PowerMarker V 3.25 and NTSYS programs.

**Identification of chromosome complements in the complex hybrids**

The presence of the chromosome complements of each diploid species in the complex hybrid nuclei was investigated by the detection of chromosome markers that included morphology of some chromosome pairs (A9 and SAT chromosomes), heterochromatin amount and distribution, and the number and localization of 18-26 rDNA and 5S rDNA (Robledo et al., 2009; Robledo and Seijo, 2010). For chromosome preparations, root apices pretreated with 2 mM L dNTPS (2.5 nM), 1.0 L water, 1.2 L genomic DNA, in a final volume of 6 L. Amplification reactions were performed in an ABI 9700 HC3 (V 6389 x V 9401) 4X x (K 3000 6 x V 6325)4X 416 4 0.96 12.50 b 0 B gAlK?hoAhe HC2 (K 30076 x V14167) 4X x (K 3000 6 x V 6325)4X 296 2 0.68 0.33 c 1 B 8gAK7hoAuct HC1 (K 30076 x V14167) 4X x (V 6389 x V 9401) 4X 554 19 3.43 65.13 a 34 B 8gAdAlB7hoAga A. hypogaea (A. hypogaea subsp. fastigiata var. fastigiata cv. IAC-Tatu-ST and cv. BR-1, A. hypogaea subsp. hypogaea cv. IAC-Runner 866 and A. hypogaea cv. IAC-Caiapó) (Table 3).
Phytopathological and entomological characterization of complex hybrids under laboratory conditions

Bioassays were performed using detached leaves (Moraes and Salgado, 1982) under controlled laboratory conditions to verify resistance to rust (*Puccinia arachidis* Speg.), fall armyworm (*Spodoptera frugiperda* J.E. Smith), and velvetbean caterpillar (*Anticarsia gemmatalis* Hübner). The four complex hybrids (three in the velvetbean caterpillar assay) and the IAC Tatu ST peanut cultivar as susceptible control were included in the assays.

Characterization of complex hybrids for resistance to rust (*Puccinia arachidis* Speg.)

Four leaves of each genotype were evaluated after 23 days of experiment. The bioassay was carried out in Petri dishes filled with a cotton layer and one blotter paper according to Moraes and Salgado (1982). The inoculation was performed using a spore solution at 100,000 spores of rust mL⁻¹. The number of pustules per leaf area (cm²) was counted. Data were analyzed using the *t*-test.

Characterization of complex hybrids for resistance to velvetbean caterpillar (*Anticarsia gemmatalis* Hübner)

One leaf of each genotype and two first-instar caterpillars were kept in a sealed Petri dish filled with a cotton layer and one blotter paper. Four replications per genotype were analyzed after a five-day experiment. The damaged leaf area was evaluated by a 1–4 damage scale (1-resistant, 2-moderate resistant, 3-moderate susceptible, 4-susceptible). Data of damaged leaf area were analyzed by the *t*-test.

Characterization of complex hybrids for the resistance to fall armyworm (*Spodoptera frugiperda* J.E. Smith)

One leaf of each genotype and two first-instar caterpillars were kept in a sealed Petri dish filled with a cotton layer and one blotter paper. Four replications of each genotype were analyzed after a five-day experiment. The damaged leaf area was evaluated by a 1–4 damage scale (1-resistant, 2-moderate resistant, 3-moderate susceptible, 4-susceptible). Data of damaged leaf area were analyzed by the *t*-test.

Table 4 - Multiplex systems, labeled primers and their respective fluorescence, base pair size, amplification temperature, and the products that were amplified and analyzed.

| Multiplex | Primer | Fluorescence | Size (bp) | Temperature (°C) | Analyzed |
|-----------|--------|--------------|-----------|-----------------|----------|
| 1         | TC3E02 | Blue         | 270-310   | 58              | X        |
|           | AC2H11 | Green        | 230-270   | 58              | X        |
|           | TC7G10 | Blue         | 110-142   | 58              | X        |
|           | TC7H11 | Blue         | 340-360   | 58              | X        |
|           | RN2C06 | Green        | 190-220   | 58              | X        |
|           | TC6E01 | Blue         | 154-186   | 58              | X        |
| 2         | TC7A02 | Blue         | 308-320   | 58              | X        |
|           | GI-338 | Green        | 240-270   | 58              | X        |
|           | TC4F12 | Blue         | 220-232   | 58              | X        |
|           | GI-832 | Green        | 200-210   | 56              | X        |
| 3         | TC11A02| Green        | 284-292   | 58              | X        |
|           | TC6H03 | Blue         | 210-228   | 58              | X        |
|           | RN22G07| Green        | 180-210   | 58              | X        |
| 4         | TC9F10 | Blue         | 286-320   | 56              | X        |
|           | TC1D02 | Blue         | 242-278   | 56              | X        |
|           | GI-342 | Green        | 210-240   | 58              | X        |
| 5         | RNO-681| Green        | 310-350   | 54              |           |
|           | TC7E04 | Blue         | 290-300   | 56              | X        |
|           | TC9F04 | Blue         | 242-278   | 54              | X        |
| 6         | AC2B03 | Green        | 296-308   | 54              | X        |
|           | TC2B09 | Blue         | 190-200   | 52              | X        |
|           | R1lF06 | Green        | 312-372   | 56              | X        |
| 7         | GI-1107| Green        | 360-384   | 52              | X        |
|           | TC1A02 | Blue         | 240-276   | 54              | X        |
|           | TC3H02 | Blue         | 280-300   | 54              | X        |
|           | TC11A04| Green        | 172-204   | 52              | X        |
|           | TC6G09 | Blue         | 132-146   | 50              | X        |
| 8         | RNO-615| Green        | 390-400   | 56              |           |
|           | TC1E01 | Blue         | 154-248   | 48              | X        |
|           | TC9C12 | Green        | 256-300   | 54              | X        |
|           | TC7C06 | Blue         | 148-176   | 52              | X        |
|           | TC11H06| Green        | 190-214   | 52              | X        |
| 9         | TC1A01 | Blue         | 202-222   | 54              | X        |
|           | TC2D06 | Blue         | 196-224   | 48              | X        |
|           | TC3E05 | Blue         | 358-370   | 48              | X        |
| 10        | RNO-681| Green        | 310-350   | 54              |           |
|           | TC1E01 | Blue         | 154-248   | 48              | X        |
|           | TC9C12 | Green        | 256-300   | 54              | X        |
|           | TC7C06 | Blue         | 148-176   | 52              | X        |
|           | TC11H06| Green        | 190-214   | 52              | X        |
| 11        | TC1A01 | Blue         | 202-222   | 54              | X        |
|           | TC2D06 | Blue         | 196-224   | 48              | X        |
|           | TC3E05 | Blue         | 358-370   | 48              | X        |
|           | RN8C09 | Green        | 260-290   | 56              | X        |

8-hydroxyquinoline for 3 h and fixed in 3:1 absolute ethanol:glacial acetic acid (Fernández and Krapovickas, 1994) were digested in 1% (w/v) cellulose (Onozuka) plus 10% (v/v) pectinase (Sigma) solution in 0.01 at 37 °C for 2 h. The meristematic cells were squashed in 45% acetic acid.

The 18S–26S and 5S rDNA loci were localized using probes isolated from genomic DNA of *A. hypogaea* (Robledo and Seijo, 2008). Pretreatment of preparations, chromosome and probe denaturation, conditions for the in situ hybridization (hybridization mixes contained DNA probes at a concentration of 2.5 – 3.5 ng/L, with a stringency to allow sequences with 80 – 85% identity to remain hybridized), posthybridization washing, blocking and indirect detection with fluorochrome-conjugated antibodies were performed according to Moscone et al. (1996) and Seijo et al. (2004). Chromosomes were analyzed and photographed with an epifluorescence microscope equipped with a digital camera system. Red, green and blue images were captured in black and white using appropriate filters for TRITC, FITC, and DAPI excitation, respectively. Digital images were combined and then processed for color balance, brightness, and contrast for uniformity across the image.
Phytopathological characterization under field conditions

Characterization of the complex hybrids

Field trials were carried out at APTA Polo Centro Norte in Pindorama, São Paulo State, Brazil (21°13′ S and 48°55′ W), where inoculum pressure for peanut phytopathogenic fungi is considered high. Three complex hybrids were evaluated: HC1 ((K 30006 x V 14167)4x x (K 30006 x V 6325)4x; HC3 (V 6389 x V 9401)4x x (K 30006 x V 6325)4x; HC4 (V 6389 x V 9401)4x x (K 30006 x G 10017)4x) and the peanut cultivar IAC Caiapô. For the Spheceloma arachidicola assay, the peanut cultivar BR-1 was also included as control.

The resistance trial was performed in four randomized blocks, with five plants per 1.5 meter rows with 0.90 spacing between rows. Seeds and seedlings were planted in pots and transplanted to the field when rooted. The evaluation was performed at 90 days. Two types of evaluation were performed: 1) using a 1-9 score scale that identifies defoliation index and damaged leaf area (Subrahmanyam et al., 1982), and 2) disease severity in the most attacked leaf of the plant. The most damaged leaves of each plant and each genotype were collected for evaluation. The evaluated diseases were late leaf spot (Cercosporidium personatum Berk and M.A. Curtis), rust, early leaf spot (Cercospora arachidicola Hori), and scab (Spheceloma arachidicola Bitanic and Jenkins). Leaves were scanned and evaluated by the analysis of the damaged area using the Image Tool® Free Software. Statistical analyses were performed using the t-test.

Characterization of the F2 progenies for foliar fungal diseases

The field assay was performed including F2 progenies, amphidiploids, complex hybrids, and A. hypogaea cultivars. Seeds were treated with Plantacol® fungicide (10 g per 100 kg seeds) and put to germinate into blotter paper, conditioned at 26 °C / 3 °C, 70 / 10% RH, and photoperiod of 12 hours. Seedlings were put in plastic cups (200 ml) with soil and manure (3:1) and kept in greenhouse conditions. Fifteen days after emergence, the plants were put in field. Plants of the F1 were planted by branches. Genotypes were placed as 3 rows of 5 plants, with five plants per 1.5 meter rows with 0.90 spacing between rows. All plots were fertilized with 8-28-16 NPK formula as 250 kg/ha dosis. The insecticide tiametoxam + lambda-cialotrina (Engeo™ Pleno, Syngenta) was sprayed every 15 days at 0.15 L/ha dosis to thrips (Moulton, 1941) (Thysanoptera: Thripidae) and rednecked armyworm (Stegasta bosquella (Chambers, 1875) (Lepidoptera: Gelechiidae) control. Pre-emergent trifluraline herbicide (2.5 L/ha) was used for weed control. Manual weeding control were done as necessary.

Foliar disease resistance evaluations were performed at 65, 80, 95 and 125 days after the transplant to the field. A 1 to 9 diagrammatic scale was used, where 1 meant no symptoms and 9 meant high disease infestation and high defoliation (Subrahmanyam et al., 1982). The severity was evaluated by the use of the area under disease progress curve (AUDPC) based on the formula AUDPC = Σ[(y1 + y2)/2](t2 - t1)], where y1 and y2 are two consecutive evaluations performed on times t1 and t2, respectively. A principal component analysis was performed based on AUDPC and the detached leaves data.

Results

Development and reproductive behavior of complex hybrids

A total of 1,596 pollinations were performed, resulting in hybrids from all the combinations, with a total of 37 individuals considered as complex hybrids (Table 2). The hybridization rate ranged from 0.68 to 3.64%. Hybrids were conserved in pots under greenhouse conditions. The percentage of stained pollen grains of the complex hybrids ranged from 0.33 to 65.13% (Table 2).

Only two combinations produced fertile hybrids: HC1 (A. ipaënsis x A. duranensis)4x x (A. gregoryi x A. lineariafolia)4x (Figure 1a) and HC2 (A. ipaënsis x A. duranensis)4x x (A. hoehnei x A. helodes)4x. The first combination produced 34 F2 seeds, while the second one generated only one F2 seed (Table 2). The combinations HC3 (A. gregoryi x A. lineariafolia)4x x (A. hoehnei x A. helodes)4x and HC4 (A. gregoryi x A. lineariafolia)4x x (A. hoehnei x A. cardenasii)4x generated F1 hybrids with higher pollen viability than HC2, but did not produce F2 seeds.

Morphological characterization of the complex hybrids

The morphological characterization showed significant differences in nine out of the 25 descriptors analyzed in the complex hybrids (Table 5). These descriptors were: length and width of the basal leaflet, and width of the apical leaflet in the main axis (MA); length of the apical leaflet and length of the stipule adnate portion in the lateral branch (LB); standard and hypanthium length, and length and width of the wing in flowers (F). The coefficients of variation among descriptors ranged from 5.9 (wing length) to 41.13% (length of the stipule adnate portion on the main axis).

Eigenvalues showed that the two first components explain 82.23% of the total morphological variation. The eight main descriptors that discriminated the complex hybrids in the principal component analysis were (in order of importance): apical leaflet length, basal leaflet length, apical leaflet width, and basal leaflet width of the main axis; apical leaflet length, width of the stipule free portion, and basal leaflet length of all branches, and finally, length of the stipule free portion on the main axis. The dispersion observed in Figure 2 evidenced a clear morphological distinctness among the complex hybrids, being HC1 and HC3 the most similar.

Mitotic chromosomes of F1 complex hybrids

All the complex amphidiploid hybrids analyzed presented 2n=4x=40. The cytological markers evidenced that the amphidiploids are composed of one complete chromo-
some complement of each of the diploid progenitors used in the initial crosses. The genome constitutions of the amphidiploids were as expected (Table 6); while HC2 (Figure 3b), HC3 (Figure 3c), and HC4 (Figure 3d) were AA K?hoB. The complements of the B genome (A. ipaënsis and A. gregoryi) were clearly detected by the absence of conspicuous heterochromatic centromeric bands. The complements of the A genome (A. cardenasii, A. duranensis, A. linearifolia) were

Table 5 - Morphological descriptors of complex hybrids.

| Descriptor                  | HC1   | HC2   | HC3   | HC4   | CV%  |
|-----------------------------|-------|-------|-------|-------|------|
| Apical leaflet length MA*   | 63.39 a| 62.12 a| 67.55 a| 48.56 a| 15.51 |
| Basal leaflet length MA     | 57.20 a| 53.68 ab| 61.93 a| 45.18 b| 13.94 |
| Apical leaflet width MA     | 31.29 a| 26.19 ab| 24.66 ab| 20.64 b| 16.08 |
| Basal leaflet width MA      | 24.59 a| 20.16 ab| 19.40 ab| 16.52 b| 17.49 |
| Petiolule length MA         | 19.21 a| 17.45 a| 20.63 a| 15.48 a| 17.41 |
| Petiole length MA           | 53.37 a| 51.98 a| 59.11 a| 53.82 a| 17.25 |
| Length of the stipule adnate part MA | 6.02 a| 11.39 a| 5.85 a| 7.43 a| 41.13 |
| Length of the stipule free part MA | 30.50 a| 27.39 a| 31.72 a| 26.23 a| 11.89 |
| Width of the stipule adnate part MA | 3.63 a| 3.61 a| 3.54 a| 3.83 a| 21.51 |
| Apical leaflet length LB    | 34.01 ab| 45.69 a| 33.02 ab| 30.34 b| 20.15 |
| Basal leaflet length LB     | 31.34 a| 36.81 a| 28.63 a| 27.79 a| 23.44 |
| Apical leaflet width LB     | 25.17 a| 26.48 a| 19.90 a| 18.10 a| 20.94 |
| Basal leaflet width LB      | 19.60 a| 20.75 a| 15.46 a| 15.26 a| 22.76 |
| Petiolule length LB         | 22.73 a| 28.22 a| 18.22 a| 23.89 a| 30.26 |
| Petiole length LB           | 12.55 a| 13.16 a| 12.67 a| 10.51 a| 17.47 |
| Length of the stipule adnate part LB | 5.91 ab| 11.39 a| 4.11 b| 4.37 b| 17.04 |
| Length of the stipule free part LB | 20.18 a| 24.59 a| 18.42 a| 15.61 a| 15.58 |
| Width of the stipule adnate part LB | 4.32 a| 3.70 a| 3.74 a| 3.97 a| 17.14 |
| Standard length             | 10.07 b| 13.20 a| 12.20 a| 12.26 a| 5.97  |
| Standard width              | 6.54 a| 7.75 a| 6.83 a| 6.77 a| 8.97  |
| Wing length                 | 7.44 b| 8.34 ab| 9.08 a| 9.08 a| 5.90  |
| Width of the wing width     | 4.91 b| 6.38 ab| 5.82 ab| 6.43 a| 11.54 |
| Inferior lip length         | 6.69 a| 6.69 a| 8.88 a| 9.14 a| 13.82 |
| Posterior lip length        | 5.57 a| 5.74 a| 5.59 a| 6.51 a| 15.52 |
| Hypanthium length           | 13.40 b| 28.50 a| 29.76 a| 34.44 a| 22.29 |

* in mm. MA = Main axis, LB = Lateral branch. CV% = coefficient of variation (in percentage)
Data with the same letters were considered similar at 5% probability
distinguished by the presence of conspicuous heterochromatic centromeric bands in all their chromosomes and by the A9 pair, which is the smallest chromosome with the largest heterochromatic band (around 40% of the chromosome length) and diffuse chromosome arms. The complement of *A. hoehnei* was also detected by the presence of heterochromatic bands in all their chromosomes, and the presence of a small chromosome but structurally different from the A9 (without diffuse arms). The patterns of 18-26S and 5S rDNA of *A. ipaënsis* and *A. gregoryi* were conserved in the complex hybrids. Most of the chromosome markers here analyzed revealed similar patterns in all the of A genome diploid species, thus the identification of species-specific chromosomes in the amphidiploids was not possible or tentative. However, the number of rDNA loci and A9 chromosomes and the pattern of heterochromatin observed was as expected in HC1, HC2 and HC4. Only in HC3 the number of observed 5S rDNA was two instead of the four expected from their parental species.

The analysis of secondary constrictions and patters of 18-26S rDNA revealed the occurrence of amphiplasty. In most cases, the extended nucleolar organizing regions observed in late prometaphase or metaphases were in chromosomes that belong to the A genome.

**Obtaining hybrids between complex hybrids and cultivated peanuts**

After 1,488 hybridizations performed in 16 different cross combinations, only six hybrid individuals were obtained (Table 3): two from crosses between cultivars of *A. hypogaea* subsp. *hypogaea* var. *hypogaea* (IAC Caiapó and IAC Runner) and HC1 (*A. ipaënsis* x *A. duranensis*)<sup>4x</sup> x (*A. gregoryi* x *A. linearifolia*)<sup>4x</sup> (Figure 1b), and four hybrids between three cultivars of *A. hypogaea* (IAC Runner, IAC Tatu ST and BR 1) and the complex hybrid HC2 (*A. ipaënsis* x *A. duranensis*)<sup>4x</sup> x (*A. hoehnei* x *A. helodes*)<sup>4x</sup>. The percentage of stained pollen grains of F<sub>1</sub> hybrids between the complex hybrids and *A. hypogaea* was relatively high and varied from 65.34 to 89.42 (Table 3). Notably, the percentages of stained pollen grains were higher with the HC2 hybrid in which the genome formula was BK?hoaAA than with HC1, which had the genome formula BBAA. The F<sub>1</sub> hybrids obtained from the crosses of cv. IAC Caiapó and IAC Runner 886 with HC1 produced 8 and 18 F<sub>2</sub> seeds, respectively. The F<sub>1</sub> hybrids generated from the crosses of cv. IAC Runner 886 and IAC Tatu ST with HC2 produced 44 and 87 F<sub>2</sub> seeds, respectively.

**Table 6 - Chromosome markers observed in the complex hybrids.** Expected markers were summarized according to published data.

| Hybrid | 45 S rDNA | 5 S | A9 + small A. hoehnei |
|--------|-----------|-----|----------------------|
|        | Expected | Observed | Expected | Observed | Expected | Observed |
| HC1    | 8 (3ipa+2greg) + (2dur+1lin) | 8 (5B +3A) | 4 (1ipa+1greg) + (1dur+1lin) | 4 | 2 | 2 |
| HC2    | 10 (3ipa)+2(dur+2 hoeh+3hel) | 10(3B+4-5A+2ho) | 4 (1ipa+1dur+1hoeh+1hel) | 4 | 2+1 | 2+1 |
| HC3    | 8 (2greg)+1(1lin+2hoeh+3hel) | 8 (2B+3A+2ho) | 4(1greg)+1(1lin+hoeh+1hel) | 2 (4) | 2+1 | 2+1 |
| HC4    | 9 (2greg)+1(1lin+2hoeh+4card) | 9 (2-3B+5A+2ho) | 4(1Greg)+1(1lin+hoeh+1card) | 4 | 2+1 | 2+1 |
Identification of hybrids via molecular characterization

Molecular markers were informative for the identification of hybrid individuals (Table 7). Plants considered hybrid on morphological and reproductive analysis presented the expected bands inherited from their respective male parents (in gray). The markers TC7A02 and TC6E0 were the most informative for the hybrid identification. Although the AC2H11 and RN2C06 markers were less informative, they also contributed to corroborate the results of the two former microsattellites.

Phytopathological and entomological characterization of complex hybrids under laboratory conditions

All the complex hybrids showed significant higher resistance to rust, fall armyworm, and velvetbean caterpillar when compared to the control IAC Tatu ST (Table 8). The resistance to caterpillars showed a similar pattern in the two instars analyzed.

Besides the lower degree of lesions observed in the hybrids compared with the *A. hypogaea* cultivar analyzed (Figure 4), lesser growth of fall armyworm was also observed when they were fed on complex hybrids leaves.

Phytopathological characterization under field conditions

Characterization of the complex hybrids

The main disease observed in the field evaluations was the late leaf spot, although lesions caused by other pathogens were also observed in different degrees. Table 9 shows that all the complex hybrids analyzed proved to be more resistant than the cultivar IAC Caipó to late leaf spot, rust, and early leaf spot. Complex hybrids were also more resistant to scab than cultivar BR 1.

Characterization of the F2 progenies

A biplot graph (Figure 5) was performed based on the AUDPC and the detached leaves data from parents, averages of F2 progenies and the outstand plant of each progeny (selected plant - sp). All F2 hybrids were located closer to the wild parents than to *A. hypogaea* cultivars. All peanut cultivars, even the most resistant one (IAC 503), were more susceptible than any wild parents, the F1 and F2 hybrid progenies.

Discussion

It is known that the amphidiploid (*A. ipaënsis* x *A. duranensis*)*4x* shows the best cross compatibility with peanut, but it is not the most resistant to diseases and pests (Michelotto et al., 2015, 2016). This is because *A. ipaënsis* and *A. duranensis* are the ancestors of cultivated peanut (Kochert et al., 1996; Seijo et al., 2004; Fávero et al., 2006; Bertioli et al., 2016). The high crossability of this AABB wild amphidiploid is highly relevant, since it can be used as a bridge for introgression of resistance genes located in other wild species that produced non-crossing amphidiploids, or which generate sterile F1 population with the cultivated peanut. Here, we demonstrated that the introgression of genes from non directly related diploid species into peanut is feasible by using a bridge AABB amphidiploid.

The hybridization assays between amphidiploids demonstrated that not all the combinations are equally compatible, since two of the four F1 complex amphidiploids produced viable F2 seeds. Moreover, it is worthy of note that the F2 of HC1 was more fertile (with 34 F2 seeds) than that of HC2 (with only one F2 seed). This difference between HC1 and HC2 is probably related to the genome constitution of the male amphidiploid. In HC1, *A. gregory* and *A. linearifolia* belong to the A and B genome, respectively, and therefore a high chromosome homologous pairing is expected in the F1 meiosis with *A. ipaënsis* (B genome) and *A. duranensis* (A genome). In HC2, while *A. helodes* is a well-known A genome species, *A. hoehnei* does not belong to the B genome, being probably of the K genome ( Custodio et al., 2013). Even though it was demonstrated that the B and K genomes have partial homeology ( Leal-Bertioli et al., 2015), it is expected that meiosis was not as regular in the case of AABB complex hybrids as discussed above. The difference in meiotic behavior discussed here is clearly reflected in the pollen stainability of the F1 of these hybrids, (65.13 in the F1 of HC1 and 0.33% in that of HC2). The sterility of F1 complex hybrids HC3 and HC4 demonstrated that even among species within the same genome there are significant different reproductive isolation barriers preventing production of viable F2.

Our results also showed a differential reproductive behavior of HC1 [( *A. ipaënsis* x *A. duranensis*)*4x* x ( *A. gregoryi* x *A. linearifolia*)*4x*] and HC2 [( *A. ipaënsis* x *A. duranensis*)*4x* x ( *A. hoehnei* x *A. helodes*)*4x*] with peanut. Interesting is the fact that in both cases the female amphidiploid came from the hybridization of the diploid progenitors of peanut. It is worthy of note that the F1 hybrids obtained showed more than 60% of stained pollen and produced fertile F2, which indirectly evidenced a good homologous pairing between the chromosomes coming from the diploid species with those of each subgenome (A and B) present in peanut. This aspect is crucial for the transmission of desirable characters from wild diploids into the peanuts subgenomes.

It is worth to mention the importance of the genetic base broadening for peanut obtained by crosses between five distinct diploid species. The most remarkable antecedent is the introgression of resistance to *Meloidogyne arenaria* (Neal) Chitwood and *M. javanica* (Treub) Chitwood in the peanut cultivar COAN. As the inheritance of this character is considered as a single, dominant gene (Bendezu and Starr, 2003), it was possible to release the first cultivar that presented a gene located in wild Arachis species and transferred to *A. hypogaea* (Simpson and Starr, 2001). The incorporation of genes from wild species of *Arachis* to *A. hypogaea*, in addition to representing a broadening of the genetic base, has contributed to the reduction of production costs, since the introduction of these genes contributes to decrease the inci-
dence of diseases and the use of pesticides, thus generating great savings for the producer (Stalker, 2017).

Hybridization and polyploidy usually have been reported as processes that induce genomic and epigenetic rearrangements (Chen, 2007; Madlung and Wendel, 2013). Only few allopolyploids remain as examples that have not undergone conspicuous chromosome rearrangements, among them is *A. hypogaea* (Seijo et al., 2018). The sum of chromosome markers here analyzed by FISH revealed that the complex amphidiploids showed a high stability in their karyotypes.

### Table 7 - Polymorphic microsatellite markers used to identify complex hybrids of *Arachis*. In the genotypes column, the materials are arranged in groups of three (or four) rows, indicating the female (F) and male (M) parents and subsequently the hybrid between these parents tested. Gray colored cells show the alleles shared between the male parent and its hybrid(s).

| Genotypes | TC7A02 | RN2C06 | TC6E01 | AC2H11 |
|-----------|--------|--------|--------|--------|
| F (K 30076 x V14167)<sup>4x</sup> | 269 | 305 | 200 | 160 | 16 | 186 | 213 | 221 | 235 |
| M (V6389 x V9401)<sup>4x</sup> | 261 | 200 | 158 | 190 | 210 | 221 |
| H HC1 | 273 | 305 | 200 | 160 | 188 | 210 | 221 | 235 |
| F (K 30076 x V14167)<sup>4x</sup> | 269 | 305 | 200 | 160 | 186 | 213 | 221 | 235 |
| M (K 30006 x V 6325)<sup>4x</sup> | 265 | 200 | 192 | 208 | 221 |
| H HC2 | 265 | 299 | 200 | 160 | 186 | 192 | 208 | 221 | 235 |
| F (V6389 x V9401)<sup>4x</sup> | 261 | 200 | 158 | 190 | 210 | 221 |
| M (K 30006 x V 6325)<sup>4x</sup> | 265 | 200 | 192 | 208 | 221 |
| H HC3 | 263 | 273 | 200 | 148 | 190 | 208 | 221 |
| F (V6389 x V9401)<sup>4x</sup> | 261 | 200 | 158 | 190 | 210 | 221 |
| M (K 30006 x G 10017)<sup>4x</sup> | 265 | 204 | 206 | 220 | 213 |
| H HC4 | 265 | 273 | 200 | 204 | 210 | 220 | 221 |
| F IAC-Tatu-ST | 289 | 299 | 188 | 200 | 160 | 202 | 221 | 251 |
| M HC1 | 273 | 305 | 200 | 160 | 188 | 210 | 221 | 235 |
| H IAC-Tatu-ST x HC1 | 263 | 299 | 200 | 160 | 186 | 194 | 221 |
| F IAC-Tatu-ST | 289 | 299 | 188 | 200 | 160 | 202 | 221 | 251 |
| M HC2 | 265 | 305 | 200 | 160 | 188 | 210 | 221 |
| H IAC-Tatu-ST x HC2 | 265 | 299 | 200 | 160 | 186 | 208 | 221 | 235 |
| F IAC-Tatu-ST | 289 | 299 | 188 | 200 | 160 | 202 | 221 | 251 |
| M HC3 | 263 | 273 | 200 | 148 | 190 | 208 | 221 |
| H IAC-Tatu-ST x HC3 | 289 | 297 | 200 | 160 | 186 | 202 | 221 | 251 |
| F IAC-Caiapó | 291 | 299 | 188 | 200 | 160 | 180 | 221 | 249 |
| M HC1 | 273 | 305 | 200 | 160 | 188 | 210 | 221 | 235 |
| H IAC-Caiapó x HC1 | 289 | 299 | 200 | 160 | 180 | 188 | 221 | 249 |
| F IAC-Caiapó | 291 | 299 | 188 | 200 | 160 | 180 | 221 | 249 |
| M HC2 | 265 | 299 | 200 | 160 | 186 | 192 | 208 | 221 | 235 |
| H IAC-Caiapó x HC2 | 291 | 299 | 200 | 160 | 180 | 188 | 221 | 249 |
| F IAC-Caiapó | 291 | 299 | 188 | 200 | 160 | 180 | 221 | 249 |
| M HC4 | 265 | 273 | 200 | 204 | 210 | 220 | 221 |
| H IAC-Caiapó x HC4 | 291 | 299 | 188 | 200 | 148 | 160 | 202 | 221 | 249 |
| F IAC-Runner 886 | 289 | 299 | 188 | 200 | 160 | 194 | 221 |
| M HC1 | 273 | 305 | 200 | 160 | 188 | 210 | 221 | 235 |
| H IAC-Runner 886 x HC1 | 261 | 299 | 200 | 160 | 188 | 194 | 221 | 251 |
| F IAC-Runner 886 | 289 | 299 | 188 | 200 | 160 | 194 | 221 |
| M HC2 | 265 | 299 | 200 | 160 | 186 | 192 | 208 | 221 | 235 |
| H IAC-Runner 886 x HC2 | 269 | 299 | 200 | 160 | 186 | 194 | 221 | 235 | 251 |
| F IAC-Runner 886 | 269 | 303 | 200 | 148 | 160 | 186 | 194 | 221 | 235 | 251 |
| M BR1 | 289 | 297 | 188 | 200 | 160 | 200 | 221 | 251 |
| H BR1 x HC2 | 265 | 299 | 200 | 160 | 186 | 192 | 208 | 221 | 235 |
| H BR1 x HC2 | 273 | 297 | 200 | 160 | 186 | 200 | 221 | 235 | 251 |

*a* = allele

Gray data means same alleles between the male parent and the hybrid
institution of this species still has to be determined, the accession used here may not be considered as belonging to the B genome as defined by Robledo and Seijo (2010). From a cytological point of view it may be better placed among the K genome species.

Concerning the morphological characterization of the germplasm here analyzed, our data demonstrated that the first two principal components explained a high percentage (> 80%) of the total variance, and that nine characters stand out as important in the phenotypic discrimination. This is in complete accordance with previously published results using simple amphidiploids (Fávero et al., 2015; Paula et al., 2017). The fact that the hybrids were morphologically more similar to the amphidiploid progenitors than to the parent A. hypogaea (except for the hybrid IAC 503 x (A. gregoryi V 6389 x A. stenosperma V 12488)4x) evidenced a high percentage of wild alleles in the progenies, supporting a significant broadening of the peanut gene pool to be used for breeding.

Leaf pests and diseases are among the most important factors that limit the economically sustainable production of peanuts worldwide. Late leaf spot and rust, if not controlled, can cause decreases of up to 70% in the production and affect peanut quality (Michelotto et al., 2013). The two peanut cultivars used in the present study were chosen because cv. IAC Caiapó is considered the most resistant cultivar to the late leaf spot and rust in the market, but susceptible to the early leaf spot; and cv. BR 1 is susceptible to scab. Despite the partial resistance in IAC Caiapó, all the interspecific hybrids were more resistant than the A. hypogaea genotypes included in both the assay done under laboratory and field conditions. Our study confirms that the resistance to these fungi present in wild diploids (Fávero et al., 2009) can be introgressed into peanut and, eventually, sources of resistance from different species can be pyramided in elite peanut varieties.

The evaluation of damaged leaf area due to foliar fungal diseases aims at the observation of how much the leaf can be attacked by foliar fungi, regardless of the pathogen. The evaluation was done by the total damaged leaf area. According to data reported by Fávero et al. (2005), in natural infection under greenhouse conditions greater resistance to late leaf spot and rust was observed in amphidiploid and segregating individuals than in cultivated peanut. In agreement with studies on resistance to leaf spot and rust, resistance to these diseases is polygenic, complex, and probably cong.
trolled by recessive genes (Dwivedi et al., 2002; Mace et al., 2006; Leal-Bertoli et al., 2010).

Due to the susceptibility to pests, such as thrips (Enneothrips flavens Moulton) and the rednecked peanut worm (Stegasta bosquella (Chambers), peanut production can be severely decreased. This susceptibility is one of the main peanut crop limitations (Lourenção et al., 2007). The use of insect-resistant peanut cultivars may have important benefits, as they keep the pests below the economic damage levels, avoid environment pollution, and reduce the chemical control costs (Lara, 1991). The bioassays here performed, using detached leaves under laboratory conditions to verify the complex hybrids resistance to fall armyworm and velvetbean caterpillar, comparing the complex hybrids with the peanut cultivar IAC Tatu ST, revealed a significant reduction in the damaged leaf area. Moreover, reduction in the growth rate of armyworm caterpillar, when they were fed with complex hybrids leaves, indicates antibiotic resistance. Campos et al. (2011) also verified this type of resistance in some peanut cultivars, but with lower intensity. According to Di Bello (2015), the runner peanut cultivars IAC 147 and IAC Runner 886 have antibiotic resistance that affects the larval survival of S. bosquella.

To conclude, it was possible to introgress wild alleles into peanut from non closely related wild diploid species (A. gregoryi, A. helodes, and A. hoehnei) by the production of complex hybrids. We demonstrate that it is feasible to introgress genes from distant wild species using complex hybrid developed from a cross between one peanut compatible amphidiploid with another one made by crossing more distant wild species.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

APF, MDM and JGS conceived and designed the experiments; APF, ARC, IJG, JGS and MDM performed the experiments; APF and MDM analyzed the data; APF, IJG and JGS contributed reagents, materials and/or analysis tools; APF, ARC, MDM, NBD and JGS drafted and wrote the manuscript. All authors approved the final version.

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