



## Crossability of *Arachis valida* and B genome *Arachis* species

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**ABSTRACT.** The peanut (*Arachis hypogaea*) is an important food crop in much of the tropical and semi-tropical parts of the world. The peanut is an allotetraploid with an AABB genome formula derived from diploids *A. duranensis* (A genome) and *A. ipaënsis* (B genome). The success of an introgression program that aims to improve cultivated varieties of the peanut depends on whether the chosen B genome species is homologous with the B genome of the peanut. While not directly involved in the origin of the peanut to the best of our knowledge, *Arachis valida* is a B genome species that could potentially be a bridge species or a source of new and different alleles, because of its resistance to diseases and pests. In this study, we investigated the crossability of *A. valida* with five other B genome species of section *Arachis*. Eight cross-combinations were made with *A. valida* and *A. gregoryi*, *A. ipaënsis*, *A. magna*, *A. valida*, and *A. williamsii*. Two hundred and forty pollinations were made yielding 61 fruit segments, 61 seeds, one abortion, and 24 hybrid plants. An analysis of the morphological characteristics and pollen viability confirmed that the plants

were hybrids. Our results indicated that higher pollen viability of hybrid plants corresponded with higher affinity between parent plants used in crossings. This conclusion corroborates much of previous research carried out by many other authors in the past.

**Key words:** Peanut; Groundnut; Wild species; Pre-breeding; Pollen; Artificial hybridization

## INTRODUCTION

*Arachis* L. is a South American genus of 81 species that thrives in Brazil, Bolivia, Paraguay, Argentina, and Uruguay, ranging from north-northeastern Brazil, just south of the equator, to the northern bank of the Rio de la Plata in Uruguay and from the Atlantic coast to the eastern foothills of the Andes (Krapovickas and Gregory, 1994; Valls and Simpson, 2005; Valls et al., 2013). Brazil has the largest number of species including 47 endemic species and 17 native species shared (Krapovickas and Gregory, 1994; Valls and Simpson, 2005; Valls et al., 2013). The peanut (*A. hypogaea* L.), also known as the groundnut, is cultivated in over 100 countries, and China, India, Nigeria and United States of America were the top producers in 2013 (FAOSTAT, 2015). The peanut is one of the most important sources of protein in the world and peanut oil is the most important peanut derived product (Burow et al., 2009). In 2012, over five million tons of peanut oil were produced (FAOSTAT, 2015). Peanuts are consumed raw, roasted, as peanut butter, in confectionary candies and as oil for cooking and food preparation.

The genus is classified into nine taxonomic sections and the cultivated peanut belongs to section *Arachis* (Krapovickas and Gregory, 1994). Within the section *Arachis*, the genomes A, B, D, F, G, K, and AB can be found based on observations of karyotype structures (Stalker, 1991; Krapovickas and Gregory, 1994; Peñaloza and Valls, 2005; Robledo et al., 2009; Robledo and Seijo, 2010; Silvestri et al., 2015). One species, that crosses with many species of section *Arachis*, *A. vallsii* Krapov. & W.C. Greg., still has an unknown genome. The diploid species characterized by the presence of one small pair of chromosomes, or “A chromosomes”, are known as the A genome species. These chromosomes have differential staining from the centromere, slight staining in the prometaphase, and one heterochromatic band at the centromeric region (Husted, 1933, 1936; Seijo et al., 2004). The A genome species can be divided into three subtypes based on the variability observed during molecular analysis, in fluorescence *in situ* hybridization (FISH) mapping, and heterochromatin detection, and over their geographic distribution: the La Plata River Basin Group, the Chiquitano Group, and the Pantanal Group (Robledo et al., 2009). The annual diploid species of section *Arachis* with a chromosome number of  $2n = 2x = 20$ , lacking pair A, was formally known as the non-A genome, or B genome species (Peñaloza and Valls, 2005; Valls and Simpson, 2005; Robledo and Seijo, 2010). Two of these species, *A. benensis* Krapov., W.C. Greg. & C.E. Simpson and *A. trinitensis* Krapov. & W.C. Greg., were re-evaluated and classified as F genome species (Robledo and Seijo, 2010), while the other three species, *A. batizocoi* Krapov. & W.C. Greg., *A. cruziana* Krapov., W.C. Greg. & C.E. Simpson, and *A. krapovickasii* C.E. Simpson, D.E. Williams, Valls & I.G. Vargas were classified as K genome species, based on molecular studies and by FISH mapping (Robledo and Seijo, 2010). Quite recently, three additional annual species of the section *Arachis* with a chromosome number of  $2n = 2x = 18$ , lacking pair A, were reclassified as G genome species (Silvestri et al., 2015). Non-A genome species have not been studied thoroughly, and their relationships among these taxa are unclear (Robledo and Seijo, 2010).

Modern peanut-breeding programs seek introgression of desirable agronomic traits, such as increased productivity and quality, resistance and/or tolerance to diseases and pests and tolerance to drought and flooding. These aforementioned agronomic traits of wild species can be transferred to peanut by crosses. Several peanut cultivars were developed in this way, such as COAN, NemaTAM, and Tifguard (Simpson and Starr, 2001; Simpson et al., 2003; Church et al., 2005; Holbrook et al., 2008).

Peanut is an allotetraploid with a chromosome number of  $2n = 4x = 40$  and an AB genome (Kochert et al., 1996). Through cytogenetic and molecular analyses, the two wild diploid species *A. duranensis* Krapov. & W.C. Greg. (provider of the A genome) and *A. ipaënsis* Krapov. & W.C. Greg. (provider of the B genome) were found to be the parents of *A. hypogaea* (Fávero et al., 2006; Seijo et al., 2004, 2007; Grabiele et al., 2012). Identifying a species with high homology to the B genome of *A. hypogaea* is important because the success of introgression breeding programs greatly depends on the choice of a species with B genome compatibility with that of the peanut (Cunha et al., 2008). Thus, understanding the relationship between various B genome species is important and justifies further intensive study. There are more than 50 available *A. duranensis* accessions (A genome). However, only one germplasm accession is available for *A. ipaënsis* (B genome), an endemic species to Bolivia. There are four other B genome species. One is an endemic species to Bolivia: *A. williamsii* Krapov. & W.C. Greg. Another is endemic to Bolivia and Brazil: *A. magna* Krapov., W.C. Greg. & C.E. Simpson. The final two are endemic only to Brazil: *A. gregoryi* C.E. Simpson, Krapov. & Valls (Valls and Simpson, 2005) and *A. valida* Krapov. & W.C. Greg. (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). These species are cytologically similar to *A. ipaënsis* because their chromosomes do not have heterochromatic bands (Robledo and Seijo, 2010). By cytogenetic and molecular analyses and crosses, *A. valida* has been found to be close to *A. ipaënsis* (Custodio, 2009; Custodio et al., 2013; Moretzsohn et al., 2013). *A. valida* has a higher number of accessions compared to the only one available accession of *A. ipaënsis*, needed to expand the genetic basis for the B genome in peanut-breeding programs. Another species is *A. magna*, which has higher intra-population heterogeneity, indicating that this species could comprise more than one taxon (Custodio et al., 2013). *A. valida* is possibly tolerant to flooding and may have disease resistance associated with excessive moisture because it grows in sites flooded during the wet season south of Mato Grosso do Sul in the Pantanal (Krapovickas and Gregory, 1994). Some accessions have resistance to early leaf spot (*Cercospora arachidicola* Hori), late leaf spot (*Cercosporidium personatum* (Berk & Curt.) Deighton), rust (*Puccinia arachidis* Speg.), and corn caterpillar infestations (*Spodoptera frugiperda* Smith) (Pande and Rao, 2001; Ramos, 2007; Fávero et al., 2009). *A. valida* is not directly involved in the origin of peanut, but it is valuable to have information about its crossability with other species, because it could potentially be used in peanut-breeding programs as a bridge species or as a source of new and different alleles. The objective of this study was to characterize the taxonomic relationships among *A. valida* and other B genome species of section *Arachis* through crosses between species.

## MATERIAL AND METHODS

### Date and location of experiments

The experiments were carried out between September 2011 and March 2013 at Embrapa Genetic Resources and Biotechnology (Embrapa Cenargen), in the Federal District of Brasília, Brazil, 15°43'51.63"S, 47°54'05.17"W, 1020 m.

## Plant material source

The seeds for the accessions were obtained from the Active Germplasm Bank of Wild Species of *Arachis* at Embrapa Genetic Resources and Biotechnology (BAG *Arachis* - Cenargen) (Table 1).

**Table 1.** Materials of *Arachis* species of section *Arachis*, abbreviations, and collector numbers; Brazilian accession code (BRA); municipality; state and country of origin; geographic coordinates: latitude (Lat) and longitude (Long) in degrees (°) and minutes ('), and altitude (Alt) in meters; genome; and ploidy level (2n).

Species	Collector* N°	BRA	Municipality - State	Country	Lat. (S)	Long. (W)	Alt. (m)	Genome	2n
<i>Arachis gregoryi</i> C.E. Simpson, Krapov. & Valls	VS 14957	040002	Vila Bela S. Trindade - MT	Brazil	15°22'	60°14'	-	B	20
<i>A. ipaënsis</i> Krapov. & W.C. Gregory	KGBPScS 30076	036234	Ipa	Bolivia	21°00'	63°25'	650	B	20
<i>A. magna</i> Krapov., W.C. Gregory & C.E. Simpson	KGSsC 30097-of	036871	San Ignacio de Velasco	Bolivia	16°22'	60°58'	370	B	20
<i>A. magna</i>	VOFSv 14707	034011	Cáceres - MT	Brazil	15°48'	58°23'	150	B	20
<i>A. magna</i>	VOFSv 14724	033839	Vila Bela S. Trindade - MT	Brazil	15°19'	60°03'	390	B	20
<i>A. magna</i>	VOFSv 14727	033847	Vila Bela S. Trindade - MT	Brazil	15°21'	60°04'	380	B	20
<i>A. valida</i> Krapov. & W.C. Gregory	VPzRcSgSv 13514	032620	Corumbá - MS	Brazil	19°07'	57°32'	70	B	20
<i>A. valida</i>	VS 15096	040410	Corumbá - MS	Brazil	19°18'	57°36'	-	B	20
<i>A. williamsii</i> Krapov. & W.C. Gregory	WiDc 1118	036897	Trinidad	Bolivia	14°48'	64°52'	-	B	20

\*Collector: Dc = D. Claire, K = A. Krapovickas, Of = F.O. Freitas, P = J. Pietrarelli, Pz = E.A. Pizarro, Rc = R.C. Oliveira, S = C.E. Simpson, Sc = A. Schinini, Sg = A.K. Singh, Sv = G.P. Silva, V = J.F.M. Valls, Wi = D.E. Williams. of: orange flower. S. Trindade: Santíssima Trindade. MS: Mato Grosso do Sul. MT: Mato Grosso.

## Seed germination

The seed germination was carried out at the Laboratory of Cytogenetics at Embrapa Genetic Resources and Biotechnology, in a TE - 40 I Tecnal germination chamber at 25°C with a 12-h light and 12-h dark photoperiod. Seeds were rolled in germitest paper moistened with distilled water. Some seeds developed a fungus and were treated with 1:5 (v/v) tetramethylthiuram disulfide (Thiram). The dormant seeds were treated with 1% Ethrel solution (2-chloroethylphosphonic acid - Etefom). After the emergence of primary roots and growth of secondary roots, seedlings were transplanted into plastic cups of soil with a 1:1 ratio red soil to sand, or a super-thin grade of expanded vermiculite. Later, seedlings were transplanted in pots with a soil mixture of 1 kg ammonium sulfate, 2 kg lime, 4 kg simple superphosphate, 700 kg red soil, and 700 kg sand.

## Hybridization technique

The crosses were performed between January 2012 and April 2012 in a greenhouse at Embrapa Genetic Resources and Biotechnology. The conventional hybridization technique was used with some adaptations described below. One male parent flower was used to pollinate one female parent flower (Nigam et al., 1990). One day before anthesis of the flower buds on the female parents, emasculation was performed by removing the anthers between 16:00 and 19:00. Pollination was performed between 7:00 and 10:00 the next day. Before pollination, water was sprayed on the emasculated flower stigmas to aid in the fixation of pollen grains. The keel and the anthers of male parents were cut and placed on the stigmas of the emasculated flowers covering it and part of the style. One to three male parent plants were used to pollinate each cross-combination. The crosses were performed as many times as needed to obtain at least three pegs for each combination. The harvest was conducted at the end of the life cycle of *A. valida*. The seeds

were germinated for analysis of successful crosses. Germination of seeds and crosses for the next generation followed the same procedures as for germination of seeds for the parents. The seeds that germinated in pots of female plants were transplanted to pots having the same soil mixture used for the pots of the parents. The success rate of crosses was calculated using the following equation:

$$\%SC = (NH / NPO) \times 100 \quad (\text{Equation 1})$$

where percentage SC is the success rate of the crossing, NH is the number of hybrids, and NPO is the number of pollinations (Fávero, 2004).

### Pollen viability analysis

A pollen viability analysis was performed by visually counting the stained pollen grains of the parent and hybrid flowers. The analysis was made by a random collection of ten flowers from each parent and a variable number from the hybrids. The flowers were collected between 7:00 and 10:00. The analysis was performed at the Laboratory of Cytogenetics at Embrapa Genetic Resources and Biotechnology. A maceration of the anthers was made with aceto-carmine:glycerine 2%. At least 500-pollen grains per sample were made by counting the number of grains present per sample at random (Guerra and Souza, 2002). The pollen grains that were well-formed and well-stained were considered viable. The average number of viable-stained pollen grains found per flower was calculated. The results are reported as a percentage of estimated viability.

### Morphological characterization

We confirmed hybridizations and conducted morphological characterizations by using the germination descriptors of each *Arachis* species, including epicotyl length, epicotyl hairiness, presence/absence of anthocyanin in the epicotyl; hypocotyl length, hypocotyl hairiness, presence/absence of anthocyanin in the hypocotyls; cotyledon and petiole lengths; and presence/absence of anthocyanin in the petioles and cotyledons (Carpes, 2010). Morphological descriptors for wild *Arachis* species were also used including the presence or absence of small, scattered, adpressed hairs on the lower surface of small leaflets; the presence, or absence of long, dense, wavy hairs on the lower surface of long leaflets; and the presence or absence of bristles on the stipules (IBPGR/ICRISAT, 1992).

## RESULTS AND DISCUSSION

### Crosses

*Arachis valida* formed hybrids with all five B genome species: *A. gregoryi*, *A. ipaënsis*, *A. magna*, *A. valida*, and *A. williamsii*. Two hundred and forty-five pollinations were performed. The number of pollinations was different among cross-combinations due to the difference of available number of male and female parent flowers. Sixty-one fruit segments and 61 seeds were harvested. Only one aborted seed was found in an *A. valida* V 13514 x *A. magna* V 14724 cross-combination. Twenty-five hybrid plants were confirmed. The hybrid plants were obtained by spontaneous germination of seedlings in pots containing female parent or by seed germination in a germination chamber (Table 2).

**Table 2.** Crossability data between *Arachis valida* and B genome *Arachis* species.

Cross combination	NPO	NFS	NS	NAB	NH	SC%	PVP%	PVH%	NSH	NABH	Small ♂/♀/H	Long ♂/♀/H	BS ♂/♀/H
<i>A. valida</i> V 13514 x													
<i>A. valida</i> V 15096	34	8	8	0	5	14.7	99.3 ± 0.4	98.4 ± 1.8	59	12	-/-	-/-	-/-
<i>A. magna</i> V 14707	5	1	1	0	1	2.0	99.4 ± 0.3	39.4 ± 0.0	10	3	+/-/+	-/-	+/-/+
<i>A. magna</i> V 14727	40	14	14	0	5	12.5	99.4 ± 0.8	29.6 ± 5.4	3	2	+/-/+	-/-	+/-/+
<i>A. magna</i> V 14724	86	16	16	1	5	5.8	99.2 ± 0.7	27.5 ± 3.6	7	1	+/-/+	-/-	+/-/+
<i>A. ipaënsis</i> K 30076	12	2	2	0	1	8.3	99.1 ± 0.6	25.3 ± 3.6	1	0	+/-/+	-/-	-/-
<i>A. magna</i> K 30097-of	30	7	7	0	4	13.3	99.5 ± 0.7	25.3 ± 6.1	20	3	+/-/+	-/-	-/-
<i>A. williamsii</i> Wi 1118	16	5	5	0	2	12.5	99.3 ± 0.7	18.6 ± 2.5	0	0	-/-/+	-/-	-/-
<i>A. gregoryi</i> V 14957	22	4	4	0	2	9.1	98.0 ± 0.9	10.5 ± 2.1	0	0	-/-	+/-/+	+/-/+
Total	245	61	61	1	25				100	21			

Collector: K = A. Krapovickas, V = J.F.M. Valls, Wi = D.E. Williams. H: hybrid, of: orange flower, ♂: male, ♀: female. Cross combination, number of pollinations (NPO), number of fruit segments (NFS), number of seeds (NS), number of aborted seeds (NAB), number of confirmed hybrids (NH), success rate of the cross (SC% = (NH/NPO) x 100), pollen viability of male parents (PVP%), pollen viability of hybrids (PVH%), number of seeds of hybrids (NSH), number of aborted seeds of hybrids (NABH), presence or absence of small, scattered, adpressed hairs on lower surface of leaflets (Small), presence (+) or absence (-) of long, dense, wavy hairs on lower surface of leaflets (Long) and presence (+) or absence (-) of bristles on stipules (BS) in parents and hybrids.

The ease of obtaining the peg did not guarantee the obtaining of a hybrid. The success rate of the crosses made was not related to the greatest number of successful pollinations. The success rate of the crossings varied even among cross-combinations made of the same species. The success rate varied between crosses of *A. valida* and four *A. magna* accessions. The rate of success calculated for *A. valida* x *A. magna* ranged from 2.0% in *A. valida* V 13514 x *A. magna* V 14707 up to 13.3% in *A. valida* V 13514 x *A. magna* K 30097-of. Over all, the highest rate of success for a cross-combination was 14.7% of *A. valida* V 13514 x *A. valida* V 15096, and the lowest rate of success for a cross-combination was 2.0% of *A. valida* V 13514 x *A. magna* V 14707 (Table 2).

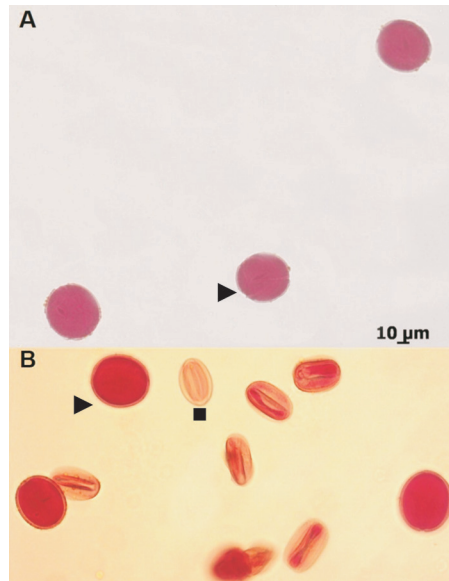
## Pollen viability

The fertility of the hybrids is one of the tools used to determine the degree of genetic isolation (Krapovickas and Gregory, 1994). In general, organisms that are closely related are more likely to be interfertile (Anderson, 1949).

Variations in pollen viability were observed in previous studies of *Arachis*. The pollen viability of 100 parents of *Arachis* species has been used in intrasectional and intersectional cross experiments and varied between 55.6 and 99.6% (Gregory and Gregory, 1979). The pollen viability of the male parents of the B genome species used in crosses with *A. gregoryi* varied between 71.9% in *A. valida* V 13514, all the way up to 99.2% in *A. magna* K 30097-of (Custodio, 2009). The range of variation was wider than in our present study because of the use of different accessions; however, the species used were the same.

The pollen viability of the parents for the present study was over 97% (Table 2), which is considered a satisfactory value, and along a success rate of over 30% for cross-combinations (Einhardt et al., 2006). Most of the pollen grains of the parents were well-formed in structure and were well-stained (Figure 1A). The pollen viability of the male parent varied between 98.0 ± 0.9% for *A. gregoryi* V 14957 and 99.5 ± 0.7% in *A. magna* K 30097-of (Table 2). The pollen viability of the female parent of *A. valida* V 13514 was 98.1 ± 1.6%. The pollen viability of the male parent of *A. valida* V 15096 was 99.3 ± 0.4% (Table 2). In this study, the highest pollen viability shown for the hybrids was 98.4% in the intraspecific cross of *A. valida* V 13514 x *A. valida* V 15096 (Table

2), whereas Custodio (2009) reported the highest pollen viability from the intraspecific cross of *A. gregoryi* V 14957 x *A. gregoryi* V 14753 as 43.6%. The lowest pollen viability of the present study was 10.5% for *A. valida* V 13514 x *A. gregoryi* V 14957 (Table 2), and Custodio (2009) reported the reciprocal cross of *A. gregoryi* V 14957 x *A. valida* V 13514 to have a pollen viability of 5.5%.



**Figure 1.** A. Pollen of female parent *Arachis valida* V 13514. B. Pollen of *Arachis valida* V 13514 x *Arachis magna* V 14707 hybrid. Arrowheads = well-formed in structure and well-stained pollen grains. Square = malformed and unstained pollen grains. Scale bar = 10 µm.

The low pollen viability of the hybrids *A. valida* V 13514 x *A. gregoryi* V 14957, *A. valida* V 13514 x *A. ipaënsis* K 30076, *A. valida* V 13514 x *A. magna* K 30097-of, *A. valida* V 13514 x *A. magna* V 14707, *A. valida* V 13514 x *A. magna* V 14724, *A. valida* V 13514 x *A. magna* V 14727, and *A. valida* V 13514 x *A. williamsii* Wi 1118 (Table 2) indicates hybrid sterility, which is a post-zygotic mechanism involved in the attempted formation of hybrids (Stebbins, 1958).

Anther dehiscence is related to the pollen viability. In wild *Arachis* species, anther dehiscence is normal when pollen viability is above 50%. Anthers open with difficulty when the pollen viability is below 50%. The anthers are indehiscent when pollen viability is around 25%; however, pollen can still be captured by bees. Anthers must be dissected to extract pollen when viability is below 15%. When viability is below 10%, it is questionable whether pollen grains, when stained, can still function during pollination (Krapovickas and Gregory, 1994). As the pollen viability of the intraspecific hybrid *A. valida* V 13514 x *A. valida* V 15096 and the hybrid *A. valida* V 13514 x *A. magna* V 14727 was higher than 50% (Table 2), the anther dehiscence of the flowers of these hybrids was normal. The hybrids *A. valida* V 13514 x *A. magna* K 30097-of, *A. valida* V 13514 x *A. magna* V 14707, *A. valida* V 13514 x *A. magna* V 14724 and *A. valida* V 13514 x *A. magna* V 14727 had pollen viability between 25.0 and 40.0% (Table 2). The anthers of flowers of these hybrids opened with difficulty and released pollen only when handled. As the pollen viability of the hybrids *A. valida* V 13514 x *A. williamsii* Wi 1118 and *A. valida* V 13514 x *A. gregoryi* V 14957 was below 20% (Table 2) the anthers of these hybrids were indehiscent.

In the pollen viability analysis of hybrids many malformed and unstained pollen grains were observed (Figure 1B). The occurrence of malformed pollen grains of these hybrids could be due to irregularities in the disjunction, e.g., the separation of chromosomes as shown in trisomy and some hybrids, the variable tendency to form quadrivalents, exchange of non-target homologous chromosomes and chromosome rearrangements (Poole, 1932).

### Hybrid seed production

All hybrids produced orange flowers and pegs. However, in the hybrid crosses, *A. valida* V 13514 x *A. gregoryi* V 14957 and *A. valida* V 13514 x *A. williamsii* Wi 1118, there was no production of fruit segments or seeds. From the other hybrids, one hundred seeds were obtained. It is likely that in 21 (Table 2), an abortion of the embryo occurred. Empty fruit segments, fruit segments with small and malformed seeds or blackened seed remnants were observed. Tallury et al. (2005) also found fruit segments with aborted seeds in crosses between *A. duranensis* with *A. benensis*, *A. cruziana*, *A. palustris*, and *A. praecox*. In the same study, in crosses between *A. batizocoi* with *A. benensis*, *A. palustris* and *A. praecox*, fruit segments with aborted embryos were recovered. Abortion of an embryo is a post-zygotic mechanism that may be caused by disharmony between genotype hybrid zygote and endosperm or maternal tissue (Stebbins 1958); this may have occurred in the *A. valida* hybrids of this study. All *Arachis* species produce pods below ground, which makes it difficult to do reproductive monitoring in interspecific crosses where the embryo often is aborted. Although pegs may appear to be healthy for several weeks after fertilization, embryos of many interspecific crosses exhibit slow growth during this period, leading to an abortion at a later stage (Tallury et al., 1995). Several processes can lead to failure to produce peanut hybrids: lack of fertilization, delayed fertilization, inability of the proembryo to resume growth after penetration of the peg into the ground, and slow growth of the proembryo. The lack of fertilization is a pre-zygotic mechanism. Late fertilization, proembryo inability to resume growth after the peg penetrates the soil, and the slow growth of the proembryo are post-zygotic mechanisms (Tallury et al., 1995).

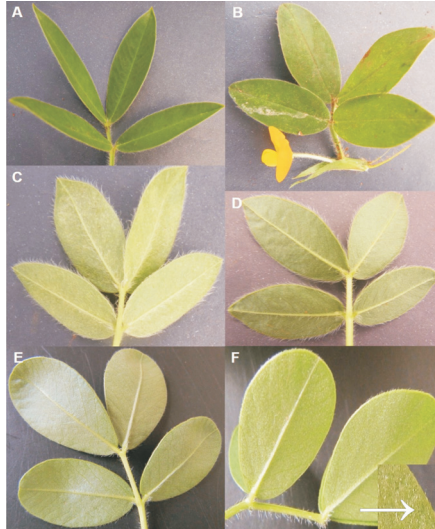
### Morphological characterization of adult plants

Leaflets of the adult hybrid plants on the main stem had the same or similar shape as the female parent, *A. valida* (Figure 2A). However, in the lateral branches, the leaflets had the same or apparently similar shape as the leaflets of the male parents (Figure 2B, C, D). Other characteristics inherited from the parents were the presence or absence of hairs and bristles.

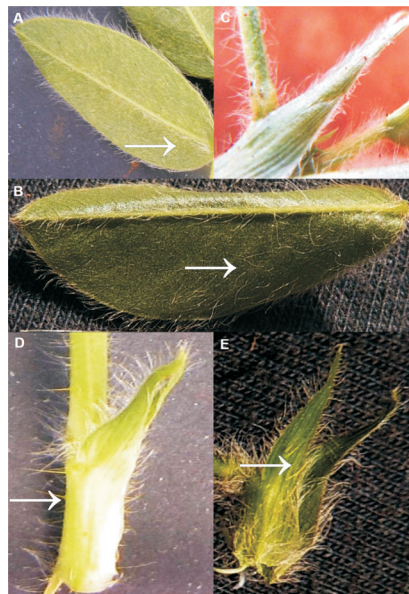
The presence or absence of small, scattered, adpressed hairs on the lower surface of leaflets was observed in parents and hybrids (Figure 2E, F). The male parent accessions of *A. magna* K 30097-of, V 14707, V 14724, V 14727, and *A. ipaënsis* K 30076 have small, scattered, adpressed hairs on the lower surface of leaflets, and as a result of crosses performed with these parents, the hybrids have these hairs as well. The female parent *A. valida* V 13514 and the male parent *A. williamsii* Wi 1118 do not have small, scattered, adpressed hairs on the lower surface of leaflets, but the hybrid *A. valida* V 13514 x *A. williamsii* Wi 1118 does have hairs (Table 2).

The presence or absence of long, dense, wavy hairs on the lower surface of leaflets was also observed in parents and hybrids. Only the male parent *A. gregoryi* V 14957 and the hybrid *A. valida* V 13514 x *A. gregoryi* V 14957 had long, dense, wavy hairs on the lower surface of leaflets (Table 2 and Figure 3A, B).





**Figure 2.** **A.** Leaf from main stem of female parent *Arachis valida* V 13514. **B.** Leaf from lateral branch of male parent *Arachis valida* V 15096. **C.** Leaf from lateral branch of female parent *Arachis gregoryi* V 14957. **D.** Leaf from lateral branch of *Arachis valida* V 13514 x *Arachis gregoryi* V 14957 hybrid. **E.** Glabrous lower surface of leaf of seedling of female parent *Arachis valida* V 13514. **F.** Adpressed hairs on lower surface of leaf from seedling of *Arachis valida* V 13514 x *Arachis magna* V 14727 hybrid (arrow).



**Figure 3.** **A.** Villous lower surface (arrow) of leaflet from lateral branch of male parent *Arachis gregoryi* V 14957. **B.** Villous lower surface (arrow) of leaflet from lateral branch of *Arachis valida* V 13514 x *Arachis gregoryi* V 14957 hybrid. **C.** Stipule without bristles on leaf from lateral branch of female parent *Arachis valida* V 13514. **D.** Stipule with bristles (arrow) on leaf from lateral branch of male parent *Arachis gregoryi* V 14957. **E.** Stipule with bristles (arrow) on leaf from lateral branch of *Arachis valida* V 13514 x *Arachis gregoryi* V 14957 hybrid.

The presence or absence of bristles on the stipules was another characteristic observed in parents and hybrids (Table 2 and Figure 3C, D, E). The hybrids that had male parents with bristles on stipules showed the same characteristic (Figure 3D, E). The male parents, *A. magna* V 14724, V 14727, *A. gregoryi* V 14957, and their hybrids had bristles on their stipules. The exception was *A. valida* V 13514 x *A. magna* V 14707. In the male parent *A. magna* V 14707 few bristles were observed on its stipules, but no bristles were seen on in the hybrid (Table 2).

### Morphological characterization of seedlings

A) *Arachis* hybrid seedlings often show characteristics of their male parents in early growth stages, e.g., the shape of the leaflets and the hairiness of the epicotyl. If the epicotyl of the male parent seedling is villous, the epicotyl of the hybrid seedling will often also be villous (Table 3 and Figure 4A, B).

**Table 3.** Morphological characterization of the seedlings of *Arachis* parents and hybrids.

Parents	Epicotyl			Hypocotyl		Cotyledons		
	Length (mm)	Hairiness	Anthocyanin	Length (mm)	Anthocyanin	Petioles length (mm)	Petioles anthocyanin	Cotyledon anthocyanin
<i>A. gregoryi</i> V 14957	26.1-126.7	villous	-/+	7.1-11.3	-/+	4.0-5.6	-	+
<i>A. ipaënsis</i> K 30076	40.2-58.9	hairs	+	8.9-29.1	-/+	4.5-7.4	-	-/+
<i>A. magna</i> K 30097-of	13.0-98.8	hairs	-/+	8.9-20.2	-/+	2.7-6.8	+	-/+
<i>A. magna</i> V 14707	21.4-60.2	hairs	-/+	10.5-30.0	-/+	2.7-6.0	-	-
<i>A. magna</i> V 14724	100.7-130.0	hairs	-/+	9.4-13.1	-/+	4.5-6.2	+	-/+
<i>A. magna</i> V 14727	50.7-121.5	hairs	+	8.8-12.3	+	4.8-6.2	-	-/+
<i>A. valida</i> V 13514	43.6-109.2	hairs	-/+	9.5-12.6	-/+	3.1-7.4	-	-
<i>A. valida</i> V 15096	17.5-148.0	hairs	-	9.0-10.4	+	2.7-4.2	-	-/+
<i>A. williamsii</i> Wi 1118	93.9-120.2	hairs	+	8.0-8.5	-	4.1-5.0	-	-
Cross combination <i>A. valida</i> V 13514 x								
<i>A. valida</i> V 15096	19.9-25.5	hairs	+	6.1-8.9	+	3.4-5.0	+	-
<i>A. magna</i> K 30097-of	55.5-66.6	hairs	+	9.8-12.8	+	2.5-6.5	+	-
<i>A. magna</i> V 14727	54.0-69.8	hairs	+	6.5-8.6	+	4.9-7.0	+	+
<i>A. williamsii</i> Wi 1118	26.7-40.5	hairs	+	5.9-7.1	+	4.4-6.3	+	-
<i>A. magna</i> V 14707	101.9	hairs	+	10.3	-	3.9-5.7	+	-
<i>A. gregoryi</i> V 14957	28.1	villous	+	6.3	+	3.9-5.3	+	-
<i>A. ipaënsis</i> K 30076	69.4	hairs	+	9.8	+	5.4-5.4	+	-
<i>A. magna</i> V 14724	8.3-38.5	hairs	+	7.3-10.3	+	4.4-6.7	+/-	+/-

Collector: K = A. Krapovickas, V = J.F.M. Valls, Wi = D.E. Williams. of: orange flower, epicotyl length in millimeters (mm), epicotyl hairiness, presence (+) or absence (-) of anthocyanin on epicotyl; hypocotyl length in millimeters (mm), presence (+) or absence (-) of anthocyanin on hypocotyl; petiole length in millimeters (mm); cotyledon length in millimeters (mm); presence (+) or absence (-) of anthocyanin in petioles; and presence (+) or absence (-) of anthocyanin in cotyledons.



**Figure 4.** A. Seedling of male parent *Arachis gregoryi* V 14957 with villous epicotyl (arrow). B. Seedling of *Arachis valida* V 13514 x *Arachis gregoryi* V 14957 hybrid with villous epicotyl (arrow).

B) In this study the length of the epicotyls of parent plants varied between 13.0 mm in *A. magna* K 30097-of to 148.0 mm in *A. valida* V 15096. The length of the epicotyls of the hybrids varied between 8.3 mm in *A. valida* V 13514 x *A. magna* V 14724 and 101.9 mm in *A. valida* V 13514 x *A. magna* V 14707.

C) A characteristic that can assist in the identification of hybrid plants is the hairiness of the epicotyl. The female parent seedling *A. valida* V 13514 had hairs on the epicotyl; however, the epicotyl was not villous like that of the male parent *A. gregoryi* V 14957 (Table 3). Hairiness was only observed in the male parent *A. gregoryi* V 14957 and the hybrid *A. valida* V 13514 x *A. gregoryi* V 14957 (Figure 4A, B).

D) The presence or absence of anthocyanin in the epicotyl varied among the parent plants. All hybrids showed anthocyanin in the epicotyl (Figure 4B).

E) The length of the hypocotyls of parent plants varied between 7.1 mm in *A. gregoryi* V 14957 and 30.0 mm in *A. magna* V 14707. The shortest length of the hypocotyl of hybrids was 5.9 mm in *A. valida* V 13514 x *A. williamsii* Wi 1118 and the longest length was 12.8 mm in *A. valida* V 13514 x *A. magna* K 30097-of (Table 3). The parents and the hybrids had no hairs on their hypocotyls. The presence or absence of anthocyanin in the hypocotyl varied among the parent plants. Only the hybrid *A. valida* V 13514 x *A. magna* V 14707 had no anthocyanin in the hypocotyl (Table 3).

F) The length of the petioles of the leaflets of the parent plants varied between 2.7 mm in *A. magna* K 30097-of, V 14707 and *A. valida* V 15096 and 7.4 mm in *A. ipaënsis* K 30076 and *A. valida* V 13514. Only the parents, *A. magna* K 30097-of and V 14724 had anthocyanin in their petioles. All the hybrids had anthocyanin in the petioles. The presence of anthocyanin in cotyledons varied among the parent plants. The cotyledons of the hybrids, *A. valida* V 13514 x *A. magna* V 14727 had anthocyanin. In the hybrids, *A. valida* V 13514 x *A. magna* V 14724 presence or absence of anthocyanin was observed (Table 3). The surface of cotyledons of all parent plants and hybrid plants was furrowed (Figure 4A, B).

Variation of the same characteristics analyzed in the parent and hybrid seedlings of this study was observed in a study of germination of *Arachis* by Carpes (2010).

G) Contrary to AB *Arachis* hybrids, BB hybrids did not have precocious germination because they belong to the same genome and therefore experienced isolation barriers. This is a post-zygotic mechanism to decrease and/or avoid the development of hybrids obtained in the nature.

### ***Arachis valida* crossability**

*Arachis valida* is a species with a B genome and has the potential to be used in a peanut-breeding program because of its resistance to diseases and pests (Pande and Rao, 2001; Ramos, 2007; Fávero et al., 2009). Data obtained in this study support *A. valida* as being of the B genome because of the hybrids we were able to produce with *A. gregoryi*, *A. ipaënsis*, *A. magna*, and *A. williamsii*. By analysis of morphological characteristics and the low pollen viability estimates for the hybrids were confirmed. These data support the theory that the higher pollen viability indicates a higher probability for affinities between species and accessions (Fernández and Krapovickas, 1994; Robledo and Seijo, 2010; Custodio et al., 2013). The accession V 13514 of *A. valida* used in this study has high resistance to early and late leaf spot (Fávero et al., 2009), which are economically important diseases in Brazil (Santos et al., 2013). *Arachis valida* V 15096, which was also used in this study, is resistant to corn caterpillar (Ramos, 2007). It will be possible to transfer these characteristics associated with known characteristics of other species of *Arachis* and to increase genetic variability in the peanut-breeding program.

## Conflicts of interest

The authors declare no conflict of interest.

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