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Successful cross of *Arachis duranensis* as female with *A. ipaënsis*

Cruzamiento exitoso de *Arachis duranensis* como madre con *A. ipaënsis*

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Summary: *Arachis hypogaea* L. originated in South America and has been taken to most of the tropical and sub-tropical parts of the world as a valuable food crop with high protein content and a source of high energy unsaturated oil. The origin of the cultivated peanut, 2n = 4*x* = 40, has been the subject of many discussions, but the primitive parents have been agreed on by most as *A. duranensis* being the A genome donor and *A. ipaënsis* the B donor; both diploids with 2*n* = 20. Whether the chromosome doubling of this hybrid occurred in a natural setting or in the garden of a hunter-gatherer-cultivator is also a subject of debate, but most likely it occurred in nature. Molecular analyses have established that *A. duranensis* was the female of the cross. Until recently no one had been successful in making and establishing plants of the cross in that direction. However, the reciprocal cross is easily accomplished and has been reported several times. The primary objective of this paper is to report the successful cross and development of hybrid plants, amphidiploids and populations from the hybrid, *A. duranensis* × *A. ipaënsis*.

Key words: A genome donor, B genome donor, hybrid, origin of cultivated peanut.

Resumen: *Arachis hypogaea* L. se originó en América del Sur y ha sido llevado a gran parte de las zonas tropicales y subtropicales del mundo como un valioso cultivo alimenticio con alto contenido de proteínas y como una fuente de aceite insaturado. El origen del maní cultivado, 2*n* = 4*x* = 40, ha sido objeto de muchas discusiones, pero la mayoría concuerda en que *A. duranensis* sería el donante del genoma A y *A. ipaënsis* el donante B; ambos diploides con 2*n* = 20. También es objeto de debate si la duplicación cromosómica de este híbrido ocurrió en un entorno natural o en el jardín de un cazador-recolector-cultivador. Los análisis moleculares han establecido que *A. duranensis* fue el progenitor femenino del cruzamiento. Hasta hace poco nadie había tenido éxito en hacer el cruzamiento y establecer plantas del mismo en esa dirección. El cruzamiento recíproco se logra fácilmente y se ha informado varias veces. El objetivo principal de este trabajo es informar sobre el éxito del cruzamiento y el desarrollo de plantas híbridas, anfidiploides y poblaciones del híbrido *A. duranensis* × *A. ipaënsis*.

Palabras clave: Donante de genoma A, donante de genoma B, híbrido, origen del maní cultivado.

Introduction

The peanut (*Arachis hypogaea* L.) has been one of the most important food legumes for peoples living in the tropical and sub-tropical regions of the world for many centuries. It also has become an important food in developed areas of the world because of its high protein, unsaturated oil content, and its desirable flavor. A large percentage of the world's peanut production is utilized for its oil which is very popular for cooking because of its long-lasting quality and nutritional value.

The genus *Arachis* most likely originated on the old Brazilian Shield in west central Brazil or northeast Paraguay sometime around

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the mid-Tertiary uplift of the Shield (Gregory *et al.,* 1980; Krapovickas & Gregory, 1994, 2007). The remnants of that eroded uplift exist even today in the area described above. Also, the three most morphologically primitive *Arachis* species described to date are still found growing in that region (Krapovickas & Gregory, 1994, 2007; Valls *et al*., 2013). Molecular estimates of that time of origin of *Arachis* have placed it around 3 million years ago (Bertioli *et al*., 2016a; Bertioli *et al*., 2016b). From the uplifted location, the genus has spread over much of Brazil, eastern Bolivia, most of Paraguay, northern Argentina, and western Uruguay. Some of this distribution has been through physical plant growth, placing seeds as much as 1 to 3 meters from the base of the mother plant. Additionally, with the geocarpic fruit of *Arachis*, running water has undoubtedly been involved in their dispersal. Documented movement by human hands has also played a significant part (Krapovickas & Gregory, 1994, 2007; Simpson *et al*., 2001; Custodio *et al*., 2005; J. F. M. Valls, personal communication).

The evolution of the genus has led to description of 83 species, to date, which have been classified into nine taxonomic sections (Krapovickas & Gregory, 1994, 2007; Valls & Simpson, 2005, 2017; Valls *et al*., 2013, Seijo *et al*., 2021). Based on the collection efforts, clearly the direction of evolution of *Arachis* has been from east to west on the South American continent. The most advanced section in the genus is Section *Arachis* which contains 33 species and includes the cultigen, *A. hypogaea*. Most of the wild *Arachis* species have 2n= 20 chromosomes. However, the cultivated peanut contains 2n= 4x= 40. Husted (1936) proposed that peanut is an allopolyploid based on the high formation of bivalents and the presence of only one pair of small chromosomes (A chromosomes), while Smartt *et al.* (1978) further named the two different chromosome sets as belonging to the A and B genomes. From the 1940's though the 1960's, several potential donors of the A-genome were proposed; most were undescribed and carried names listed as *nom. nud*. Until the mid-1970's, only one non-A group of plants in the *Arachis* section was known to exist, and it was later described and named *A. batizocoi* Krapov. & W.C. Greg. Smartt *et al*. (1978) proposed that this species might be the B-genome donor to the cultivated peanut. During the early 1970's, this author had initiated an introgression program with *Arachis* using this only known non-A as the B-genome parent to transfer leafspot resistance into the cultigen (Simpson, 1991). In 1976, extensive collection of *Arachis* germplasm was initiated and funded, in part by the International Board for Plant Genetic Resources (IBPGR) (Krapovickas & Gregory, 1994 page 7). Several new non-A genome materials were collected over the following years, providing more options to consider as the B-genome donor for *A. hypogaea* (Krapovickas & Gregory, 1994, 2007; Valls & Simpson, 2005; Robledo & Seijo, 2010; Seijo *et al*., 2021).

Kochert *et al*. (1991) proposed that *A. duranensis* Krapov. & W. C. Greg. and *A. ipaënsis* Krapov. & W. C. Greg. were the A- and B-genome donors to the cultivated peanut. Since then, numerous molecular studies have supported these two species as the likely genomic parents to the cultigen (Kochert *et al.,* 1996; Burow *et al*., 2001; Seijo *et al.,* 2007; Grabiele *et al*., 2012; Bertioli *et al*. 2016a, b).

During the development of data to identify and separate various groups of wild peanut accessions into species, plant morphology played a major part, but cross compatibility also proved to be a valuable tool in this difficult work (Gregory & Gregory, 1979; Singh, 1988; Simpson, 1991; Singh & Smartt, 1998). As time progressed, isozymes proved useful (Krapovickas, 1969; Lu & Pickersgill, 1993). Recently molecular analyses have become widely used. A major contribution of early molecular work was the capability to identify the female parent in a cross between two species (Kochert *et al.*, 1996; Grabiele *et al*., 2012, and others). Molecular analyses determined that the female in the cross which formed cultivated peanut was *A. duranensis* (Kochert *et al.*,1996; Seijo *et al*., 2004; Robledo *et al*., 2009). Herein lies the problem, which is the focus of this paper, *i.e*., until recently no one had successfully made nor documented making the cross with *A. duranensis* as the female. Gregory & Gregory (1979) reported on diallel crosses of 91 wild and cultivated *Arachis*

parents, and other researchers, including the author, have conducted several crossing programs involving materials collected since 1976 (many results unpublished).

The author's program has attempted the cross, *A. duranensis* × *A. ipaënsis*, several times from 1980 to 2010 without success, using the accession K 7988 for *A. duranensis* and K 19455 and later the KGBPScS 30076 for *A. ipaënsis*. The author successfully made the reciprocal hybrid several times (unpublished). Fávero *et al*. (2006) reported the hybrid, *A. ipaënsis* × *A. duranensis*, and produced several breeding lines after crossing the chromosome doubled progeny with each of four botanical varieties of the cultigen, *A. hypogaea.* Fávero did not make specific comparisons of the amphidiploid progeny to *A. monticola* or *A. hypogaea*; such activity was not a part of her objectives (personal communication, 2007).

Grabiele *et al*. (2012) reported on molecular studies of the *A. duranensis* materials of northwest Argentina, and concluded that materials from the Rio Seco Valley were the most likely donors of the A-genome to the cultigen, *A. hypogaea*. More specifically, their accession Se 2741 (corresponding to our collection from the same population, KGBPScS 30067), was identified as the specific accession being the A-genome donor. Studying the publication of their work, I determined that in 1977 our team had collected from virtually the same sites as those reported by Grabiele *et al*. (2012). Thus, crosses were attempted with five collections as the female and *A. ipaënsis* (KGBPScS 30076) as the male (Table 1). An oral presentation on the hybrids was made (Simpson, 2017) and a detailed paper was published (García *et al*., 2020) adding valuable information about this difficult cross. The hybrids are herein reported.

Materials and Methods

All plants in this study were grown in greenhouses to maintain purity of our germplasm. Outside grown wild *Arachis* are subject to numerous cross-pollination events virtually every summer day at our location (32°14'42"N, 98°11'49"W, 400 m).

The *A. duranensis* accessions used in these crosses are shown in Table 1. We included a check accession from outside the Rio Seco Valley, K 7988. The male of the crosses was one plant of *A. ipaënsis* (KGBPScS 30076, PI 468322).

Female plants were grown in ½ bushel fruit baskets in a sandy loam soil mix that is ca. 75% sand. Commercial inoculum was added to the

Table 1. Data from five attempted crosses of *Arachis duranensis* × *A. ipaënsis*

Crosses		Plants	Pegs	Fruits	Seeds
Cross 1	K30065 × K30076	A	2	3 segs	3 normal
		B	2	1 seg	1 normal
Cross 2	K38904 × K30076	A	3	5 segs	5 normal
	(K38904 = K30066)	B	3	5 segs	4 normal
Cross 3	K30067 × K30076	A	4	6 segs	6 normal
		B	2	4 segs	4 normal
Cross 4	K30068 × K30076	A	3	2 segs	2 normal
		B	2	4 segs	2 normal
Cross 5	K7988 × K30076	A	1	1 seg	aborted early
		B	1	1 seg	aborted early
				Total seed	**27**

mix. Seeds were pre-germinated and transplanted to baskets 3 to 4 D (days) after germination. Plants initiated flowering ca. 25 DAP (days after pollination). All flowers that were not emasculated for crossing were picked as buds before daybreak (beginning of anthesis) each morning. Emasculations were made between 7:30 and 9:00 PM, CDT (central daylight time). Pollinations were made between 7:00 and 8:00 AM the following morning. The emasculation/pollination technique is one adapted from a former student from Senegal, whereby none of the petals are removed and the bud is closed promptly after emasculation. The treated buds are then either covered with a moist paper towel or misted with an atomizer. Pollination the following morning was accomplished by cutting the keel, including anthers and stigma, deep into the bud of the male flower. After opening the petals of the emasculated (female) flower, the male keel including anthers and stigma, was fitted over the stigma of the emasculated bud. The pegs emerged in ca. 5-8 days, and a nylon string was tied around them and fixed to a wood stake with the date and cross ID (identification). Seeds were harvested at ca. 55 days after pollination. Breaking dormancy on the hybrid seeds was attempted with powdered ethylene compound and/or ethylene gas.

Chromosome doubling of hybrids was accomplished with colchicine using a technique that was described by Banks (1977). A three-day-old seedling, ca. 5 cm long, was inverted in a vial and immersed to the base of the cotyledons in a 0.2% concentration of colchicine. The vial was stoppered and placed in a chamber maintained at 30 °C for 8 hr. The treated seedling was gently washed for ca. 0.5 hr., then planted.

Pollen counts were made on the resulting amphidiploid by staining mature pollen with aceto-carmine mixed with glycerin, v/v. Full counts consisted of three flowers counted on different days with 500 random grains counted per flower, for a total of 1,500 (Gregory & Gregory, 1979).

A total of 17 plant traits were measured (Table 3) on the 21 plants studied (Table 2), the measurements were made with a hand-held digital caliper.

Results

Table 1 shows the numbers of pod segments harvested from the four crosses. All four of the accessions of *A. duranensis* collected from the Rio Seco Valley of NW Argentina produced seed. The total from all crosses was 27. Embryos of the attempted crosses with the K 7988 accession all aborted early in the developmental stages. It has been common practice in the author's breeding program to allow seed, especially interspecific

Table 2. Identity of the *Arachis* plants in Fig. 2 and the pods in Figs. 3 and 4.

Figure Code	Accessions
A - M	Plants growing from the 13 seed harvested from the new amphidiploid from the *A. duranensis* × *A. ipaënsis* cross
N	ScVn21769, *A. monticola*
O	KG30062, *A. monticola*
P	KG30063, *A. monticola*
Q	Valls 12549, *A. hypogaea*
R	US 224 = PI 475871, *A. hypogaea*
S	Cultivar 'Spantex' *A. hypogaea*
T	US 517 = PI 476209, *A. hypogaea*
U	US 217 = PI 475364, *A. hypogaea*

Table 3. Measurements recorded on 13 F2C2 amphidiploid plants and 11 check accessions.

Code	Trait
A	Mainstem height in cm
B	Coty-lat (cotyledonary lateral) length in cm
C	Coty-lat 5th internode length in cm
D	Mainstem 5th leaf, apical leaflet length in mm
E	Mainstem 5th leaf, apical leaflet width in mm
F	Mainstem 5th leaf, basal leaflet length in mm
G	Mainstem 5th leaf, basal leaflet width in mm
H	Coty-lat 5th leaf apical leaflet length in mm
I	Coty-lat 5th leaf apical leaflet width in mm
J	Coty-lat 5th leaf basal leaflet length in mm
K	Coty-lat 5th leaf basal leaflet width in mm
L	Mainstem 5th leaf petiole length in mm
M	Mainstem 5th leaf rachis length in mm
N	Coty-lat 5th leaf petiole length in mm
O	Coty-lat 5th leaf rachis length in mm
P	Mainstem 5th leaf free stipule in mm
Q	Coty-lat 5th leaf free stipule in mm

hybrid seed, to rest for a minimum of four months after harvest (a recommendation from Drs. W. C. and M. P. Gregory, personal communication, ca 1975). When it was decided to germinate some of the seed for colchicine treatment, all attempts to break dormancy were unsuccessful for more than 25 months. The dormant seeds remained firm in the "rag-dolls" in the germinator, and seeds were re-treated numerous times with some form of ethylene (gas, liquid, powder), without success. However, after 26 months, two of the seeds germinated following an ethylene treatment. The two seedlings were treated with 0.2% colchicine and one amphidiploid was obtained (Fig. 1). The second plantlet did not double and was used for other studies (see Fig. 4). García *et al*., 2020, reported a similar dormancy issue with the hybrid seeds they made and studied.

Pollen counts of the amphidiploid were 98% stained, and pegs were produced on the two cotyledonary laterals and one secondary lateral (See Discussion below).

As a precaution, cuttings were made of the amphidiploid as soon as sufficient growth had occurred. During the second month of growth, a problem developed in the R/O (Reverse Osmosis) watering system that caused high concentrations of salt to enter the water. With the action of the salt, the amphidiploid started to deteriorate, which brought the problem to our attention. However, it was too late, and the plant died in spite of our efforts. The cuttings were also affected and eventually, died as well. Before death of the plant and cuttings, we were successful in obtaining 13 viable seeds.

The 13 seeds were planted for study and for comparison to three accessions of *A. monticola*, four primitive accessions of *A. hypogaea*, and the most primitive cultivar from Texas peanut production history. These 21 accessions are identified in Table 2 and are shown in Figures 2 and 3.

Measurements of the 17 plant traits (measurements not shown, only the means and their analysis, Table 4) taken at 75 DAP for comparison between the new amphidiploids and the more evolved tetraploids showed that a majority of the comparisons were not significantly different. For example, the main axis of the two groups were essentially equal; whereas, the cotyledonary laterals were significantly different in length, with the new amphidiploids having a much longer lateral branch. This is as expected in our experience with interspecific hybrids. Another interesting comparison is leaflet length and width. Leaflet length was not significant, but width was significant. Again, as one would expect, no great differences in leaflet length were observed, but greater variability has evolved in leaflet width, with the most primitive accessions containing narrower leaflets.

The fruit characteristics of the new amphidiploids closely resembled those of the *A. monticola* accessions (Fig. 3). However, significant evolution of pod traits has occurred in selections of the *A. hypogaea*-like derivatives made by early cultivators (See Fig. 3 Q, R, S, T and U).

Also, first flower date was recorded, and pollen count percentages were made (data not presented). Pod segments were harvested from each of the plants (Fig. 3).

Fig. 1. Amphidiploid *Arachis* plant from cross, *A. duranensis* × *A. ipaënsis*, with chromosome number doubled to 2n= 40 (Photo by J. M. Cason).

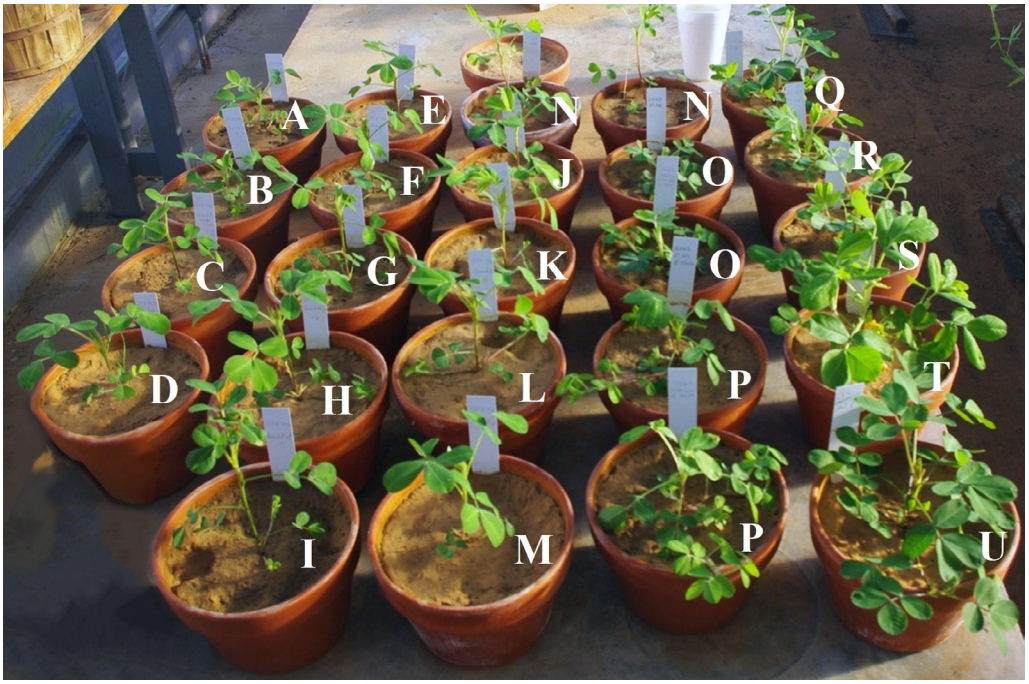

Fig. 2. Comparison of 13 amphidiploid plants (A-M) with three *Arachis monticola* accessions (N, O, P), four primitive landraces of *A. hypogaea* (Q, R, T, U) and 1 primitive cultivar (S). See Table 2 for identification of accessions.

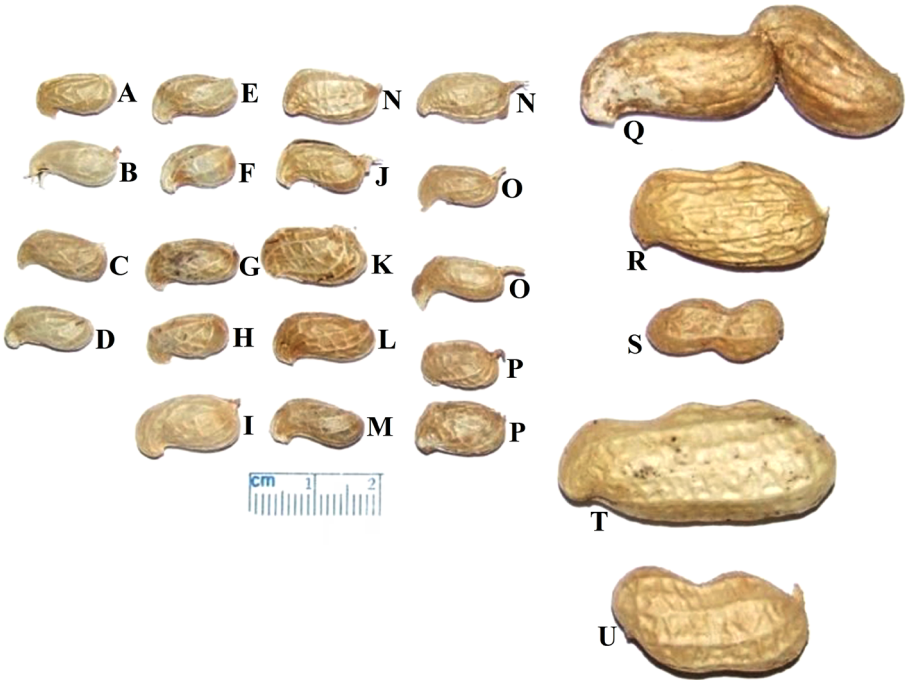

Fig. 3. Fruits (pods) from the 24 plants shown in Fig. 2. See Table 2 for identification of accessions. Note reticulation of 13 new amphidiploids (A-M) as compared to three accessions of *Arachis monticola* (N, O, P).

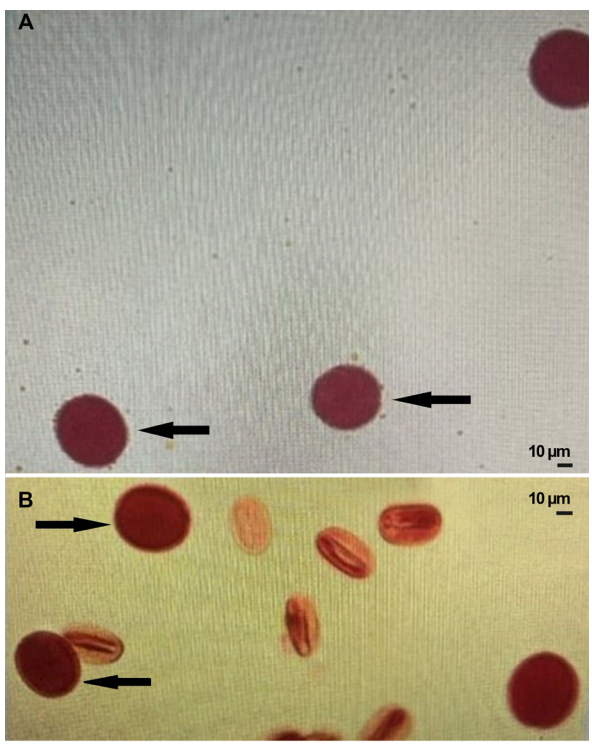

Fig. 4. Pollen grains. A: *Arachis monticola* (KG 30062). B: Diploid hybrid (2n= 20), *A. duranensis* × *A. ipaënsis*. Large well stained grains in B (arrows) are the same size as the grains of the tetraploid *A. monticola* (A), indicating the grains are unreduced.

Table 4. Statistical data on 13 amphidiploid and 11 check accessions.

Measured trait[a]	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Total mean	17.6	45	3.7	53.1	28.3	48.6	24.7	38.7	27.2	34.1	22.7	52.5	16.8	24.7	12.9	27.3	18.8
Mean of Amph's	18.7	57.4	4.6	53.7	31.3	49.1	26.7	42.9	28.9	37.7	23.7	54.7	18	32.7	13.9	26.7	20.7
Mean of Checks	16.4	32.6	2.8	52.5	25.3	48	22.5	34.4	25.5	30.5	21.7	50.3	15.6	16.8	12	28.1	16.9
Statistical Sign.	NS	*	*	NS	*	NS	*	NS	*	NS	NS	NS	NS	*	NS	NS	*
F value	0.5891	0.0009	0.0007	0.0187	0.0002	0.0218	0.0058	0.0282	0.0033	0.0267	0.2301	0.0418	0.0515	0.0002	0.0411	0.0966	0.0019

References: [a] See Table 3 for identity of traits.

Discussion

The introgression program initiated in 1973, using *A. batizocoi* (K 9484) as the B-genome parent in the crosses which seemed to be logical because it was the only species without the small "A" pair of chromosomes identified by Husted (1936). The introgression work proved to be somewhat successful, with no less than six commercial cultivars being released containing genes from that effort (Simpson, 1991). However, following the work of Kochert *et al*. (1991) and others, it became obvious that the 13 backcrosses preceding the first cultivar release had compensated for the difference between *A. batizocoi* and *A. ipaënsis*.

Gregory & Gregory (1979) did not report the cross *A. duranensis* (K-7988) × *A. ipaënsis* (K-19455) as being successful, but Krapovickas & Gregory (1994, 2007) reported that the reciprocal was easily made. The author had an opportunity to study unpublished Gregory & Gregory data pertaining to the *A. duranensis* × *A. ipaënsis* cross. From the Gregory's 1976 crossing program 46 seed segments were harvested from the *A. duranensis* × *A. ipaënsis* cross, but only one young plant was noted as hybrid, and that was a visual determination. No evaluation data such as pollen counts or plant measurements were shown. My assessment is they made the cross but did not get the evaluation finished before retirement.

The dormancy of wild *Arachis* species hybrids exhibits a wide range of variability depending on the parents involved. In the author's work over the past 55 years with *Arachis*, it has been a common occurrence for interspecific hybrids to express no dormancy and to germinate 45 to 50 days after pollination. However, some previous interspecific hybrids have exhibited dormancy periods of up to 24 months, especially if *A. duranensis* is one of the parents (unpublished data). Thus, the dormancy of the *A. duranensis* × *A. ipaënsis* hybrid seeds was not uncommon, but the length of the dormant period was perplexing.

The pollen count of the amphidiploid of almost 100% indicates that the doubled chromosome complement was complete, and the progeny should breed true in subsequent generations.

The loss of our original amphidiploid was a significant occurrence because all that remains of the plant is two herbarium specimens. The 13 seeds were harvested and planted for this study. Populations developed from these seed will continue to be studied.

The primary objective of this research study was to determine if the cross, *A. duranensis* × *A. ipaënsis*, could be made. If so, this question followed: did this cross give rise directly to *A. monticola* or to *A. hypogaea*? Morphologically, our results indicate a primitive *A. monticola* plant was produced, but the fruits were similar to modern-day *A. monticola*. Therefore, the author is convinced that the original cross of *A. duranensis* × *A. ipaënsis* occurred in nature and spontaneously doubled its chromosomes by fertilization of an unreduced egg nucleus by an unreduced pollen grain nucleus (See below and Fig. 4). This union resulted in a primitive form of *A. monticola* with plants larger than the *A. monticola* we collect today. However, the fruits of the new amphidiploid and today's *A. monticola* are almost identical (Fig. 3).

This author has found that the accessions of cultivated peanut tested in other studies were not able to survive in nature without man's assistance. The plants produce too many fruits in a small area and choke themselves out in 2 to 4 years (unpublished data from several trials with *fastigiata* and *hypogaea* subspecies). This supports the theory of man's involvement in the development of the cultigen, *A. hypogaea.*

The pollen counts of the F_1 from the *A. duranensis* × *A. ipaënsis* cross revealed a likely source of the 40-chromosome progeny from a diploid (20 × 20) cross. Counts of 1500 grains showed that 15.4% of the pollen grains in this plant were approximately twice the size of the other grains and had a fully stained, viable appearance (Fig. 4A from *A. monticola* [KG 30062], Fig. 4B from diploid *A. duranensis* × *A. ipaënsis*). This indicates that approximately 1 in 6 of the pollen grains are unreduced. Since this phenomenon has been observed in the pollen, it is plausible that it also occurs in the nuclei of the ovule which forms the egg as well (See references below). In a random occurrence, when an unreduced pollen sperm nucleus fertilizes an unreduced egg nucleus, the result is an unreduced embryo with, in this

case, 40 chromosomes. This phenomenon is not uncommon in the genus *Arachis* (D'Cruz & Chakravarty, 1961; Kumar *et al*., 1957; Simpson & Davis, 1983). Also, García *et al*. (2020), presented detailed analyses of this possible process in their *A. duranensis* × *A. ipaënsis* progeny.

Conclusion

The two tetraploid species in section *Arachis* of the genus *Arachis*, *A. monticola* and *A. hypogaea*, probably originated from the same cross between *A. duranensis* and *A. ipaënsis*. No doubt, the original cross occurred in nature because the resulting plant(s) would have been sterile and, thus, rogued out if it happened in the garden of a hunter/gatherer/cultivator.

The two-year dormant period of the hybrid seed in our study presents some amazement that the event occurred at all. After the chromosome doubling occurred, the amphidiploid seed germinated and grew into a primitive form of *A. monticola*. This statement is based on the doubled plant observed in this study. The original doubled hybrid(s) would likely have been recognized as something different from the *A. duranensis* and/or *A. ipaënsis* plants growing at the time, whether in nature or in the cultivator's plantings. Information indicates that human hands played a role in early development of both *A. monticola* and *A. hypogaea* (Krapovickas & Gregory, 1994, 2007). So, if the chromosome doubling occurred in nature, once discovered, the primitive *A. monticola* probably soon appeared in the cultivator's plantings. A widespread distribution, followed by 3500 to 3800 years of human selection and environmental pressures, led to the cultivated peanut that we know now.

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