



PRODUCTION AND CHARACTERIZATION OF AMPHIDIPOIDS FROM HYBRIDS BETWEEN *ARACHIS IPAËNSIS* AND *A. DURANENSIS* (FABACEAE): IMPLICATIONS FOR PEANUT IMPROVEMENT

PRODUCCIÓN Y CARACTERIZACIÓN DE ANFIDIPOIDES A PARTIR DE HÍBRIDOS ENTRE *ARACHIS IPAËNSIS* Y *A. DURANENSIS* (FABACEAE): IMPLICANCIAS PARA EL MEJORAMIENTO DEL MANÍ

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SUMMARY

Background and aims: *Arachis hypogaea* is an allotetraploid species of global economic importance; however, its narrow genetic base renders it vulnerable to pests and diseases. In contrast, wild diploid *Arachis* species possess valuable resistance alleles, but ploidy differences limit their direct use in breeding. Synthetic amphidiploids overcome this barrier, however, BBAA amphidiploids compatible with peanut are not available in Argentina. This study aimed to develop amphidiploid individuals suitable for national pre-breeding efforts.

M&M: Two chromosome duplication protocols were evaluated in hybrids [*A. ipaënsis* × *A. duranensis*]^{2x} based on partial or complete immersion of cuttings in a 0.2 % colchicine solution. Ploidy level was verified by flow cytometry and genome composition was confirmed by DAPI banding. Pollen viability was estimated via acetocarmine staining. Fruit characterization was performed using Fiji software.

Results: Flow cytometry confirmed that only protocol II yielded tetraploid individuals. DAPI banding revealed the expected BBAA genome configuration, and restored fertility was demonstrated by high pollen viability and fruit production. Morphologically, amphidiploids showed vigorous growth and increased fruit size.

Conclusions: Fertile BBAA amphidiploids were developed as a strategic resource for peanut pre-breeding in Argentina. Additionally, it will support studies on polyploid genome stabilization, and trait expression following hybridization and chromosome duplication in *Arachis*.

KEY WORDS

Arachis, chromosome doubling, cytogenetics, pre-breeding, synthetic allotetraploids.

RESUMEN

Introducción y objetivos: *Arachis hypogaea* es un alotetraploide de importancia económica mundial, cuya base genética estrecha lo hace vulnerable a plagas y enfermedades. Las especies silvestres diploides de *Arachis* poseen alelos de resistencia, pero las diferencias de ploidía limitan su uso directo en el mejoramiento. Los anfidiplóides sintéticos superan esta barrera, sin embargo, anfidiplóides BBAA compatibles con el maní no están disponibles en Argentina. El objetivo de trabajo fue desarrollar anfidiplóides para los programas nacionales de pre-mejoramiento.

M&M: Se evaluaron dos protocolos de duplicación cromosómica en híbridos [*A. ipaënsis* × *A. duranensis*]^{2x} basados en inmersión parcial o completa de estacas en solución de colchicina al 0,2 %. El nivel de ploidía se determinó mediante citometría de flujo y la composición genómica por bandeo DAPI. La viabilidad se estimó mediante tinción con aceto-carmin. La caracterización de frutos se realizó con el software Fiji.

Resultados: La citometría de flujo confirmó que solo el protocolo II produjo individuos tetraploides. El bandeo DAPI reveló la configuración genómica BBAA, y la fertilidad se evidenció por la alta viabilidad polínica y producción de frutos. Morfológicamente, los anfidiplóides mostraron crecimiento vigoroso y aumento del tamaño de los frutos.

Conclusiones: Se desarrollaron anfidiplóides BBAA fértiles como recurso estratégico para el pre-mejoramiento del maní en Argentina. Además, servirá de apoyo a estudios sobre estabilización de genomas poliploides, y expresión de rasgos post hibridación y duplicación cromosómica en *Arachis*.

PALABRAS CLAVE

Alotetraploides sintéticos, *Arachis*, citogenética, duplicación cromosómica, pre-mejoramiento.

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INTRODUCTION

Peanut (*Arachis hypogaea* L.) is a major legume crop of global economic and nutritional relevance. It provides high levels of plant-based protein and oil, along with essential micronutrients, and ranks among the top three oilseed crops worldwide (Akram *et al.*, 2018; Mingrou *et al.*, 2022). The species is an allotetraploid ($2n = 4x = 40$, AABB) that originated in South America via hybridization between *A. duranensis* (AA) and *A. ipaënsis* (BB), followed by chromosome duplication (Kochert *et al.*, 1996; Seijo *et al.*, 2004, 2007; Bertoli *et al.*, 2016, 2019). Currently, peanut is cultivated in more than 100 countries, with global production reaching approximately 51.7 million tons (USDA Foreign Agricultural Service, 2025). In Argentina, the production is concentrated in the central region, particularly in the Province of Córdoba, which accounts for over 74 % of peanut farms and 90 % of the agroindustrial sector (SISA, 2023). Although national production volumes are modest, approximately 80 % of the harvest is exported, positioning peanut as a strategic export commodity in the country's agricultural economy.

Currently, peanut cultivation is constrained by *A. hypogaea* narrow genetic base, which limits its adaptability and resistance to both biotic and abiotic stresses. One of the most severe threats in Argentina is peanut smut, caused by *Thecaphora frezii* Carranza & Lindquist, a soil-borne fungus (Marinelli *et al.*, 2008; Rago *et al.*, 2017). The pathogen colonizes the peg and pod in the R2-R3 developmental stages of peanut fruit, invades the tissue up to the seed coat and colonize mainly the tegument, where the hyphae initiate teliosporogenesis, leading to hypertrophy of tissues and the formation of carbonaceous mass of teliospores that replaces the seed (Romero *et al.*, 2025). Smut disease was first identified in Argentine commercial peanut fields in 1995 in the Province of Córdoba (Marinelli *et al.*, 1995) and has since rapidly spread across the entire peanut-producing region of the country. The extent of damage caused by the disease is strongly influenced by both environmental factors and crop management strategies, since teliospores remain viable in the soil for at least six years, contributing to high levels of field persistence (Paredes *et al.*, 2022, 2024). Disease incidence in some regions can exceed 50 %, with yield losses reaching up to 35 % (Oddino *et al.*, 2010; Paredes *et al.*, 2016; Rago *et al.*, 2017). In recent years, smut incidence increased from 1.66 % in

2015 to 11.47 % in 2020, resulting in estimated losses of 27,419 metric tons —about US\$14.15 million or 3.15 % of national peanut production (Paredes *et al.*, 2016, 2022).

Although management strategies such as crop rotation, tillage, and fungicide applications have been implemented, none offer complete control of the disease (Marraro *et al.*, 2009; Astiz Gasso *et al.*, 2010; Paredes *et al.*, 2024). The recent development of promising smut-resistant cultivars (Oddino *et al.*, 2023), highlights the strategic importance of broadening the genetic base through traditional genetic crossing with wild species (de Blas *et al.*, 2021; Ballén-Taborda *et al.*, 2022) for national and international breeding programs. In this sense, the genus *Arachis* (Fabaceae), endemic to South America, comprises 84 described species distributed across nine taxonomic sections based on morphology, cytogenetic, and molecular data (Krapovickas & Gregory, 1994; Valls & Simpson, 2005, 2017; Valls *et al.*, 2013; Santana & Valls, 2015; Seijo *et al.*, 2021, 2025). Among these, section *Arachis* is the most diverse, comprising 33 species with two basic chromosome numbers ($x = 9$ and $x = 10$) and two ploidy levels: diploid ($2n = 2x = 20$ or 18) and tetraploid ($2n = 4x = 40$) (Fernández & Krapovickas, 1994; Lavia, 1996, 1998, 2000; Peñaloza & Valls, 2005; Valls & Simpson, 2005; Seijo *et al.*, 2021, 2025). Most species in this section are $2n = 20$ and exhibit diverse genome types, including AA, BB, DD, FF, and KK, while the three species with $2n = 18$ belong to the GG genome group (Stalker, 1991; Robledo *et al.*, 2009; Robledo & Seijo, 2010; Silvestri *et al.*, 2015). The only natural tetraploids are *A. hypogaea* L., the cultivated peanut, and its wild ancestor *A. monticola* (Smartt *et al.*, 1978).

Wild *Arachis* species constitute a valuable source of alleles for resistance and other important traits for crop improvement (Stalker, 2017; de Blas *et al.*, 2019; Rosso *et al.*, 2025). However, despite their recognized potential, these Crop Wild Relatives (CWRs) remain underutilized in peanut breeding programs, largely due to biological constraints and insufficient cross-compatibility data (García *et al.*, 2024). Their effective use in peanut breeding must overcome ploidy differences, which prevent direct hybridization. To address these differences, synthetic amphidiploids obtained through chromosome doubling of interspecific diploid hybrids (Simpson, 2001), serve as fertile bridges for gene introgression into *A. hypogaea*. Multiple amphidiploids have been

developed using interspecific hybrids from wild species, some of which exhibit promising features for crop improvements (Simpson, 1991; Fávero *et al.*, 2006, 2015a; Mallikarjuna *et al.*, 2011; Leal-Bertioli *et al.*, 2015, 2017; de Blas *et al.*, 2019; Chu *et al.*, 2021; Gao *et al.*, 2021; Simpson, 2024). Among them, the amphidiploid derived from [*A. ipaënsis* × *A. duranensis*]^{2x}, the two wild species most closely related to peanut, is considered one of the most cytogenetically stable and compatible with *A. hypogaea* (Fávero *et al.*, 2006; Simpson, 2024). Although this genotype shows moderate resistance levels compared to other combinations, it has proven to be an ideal platform for developing complex hybrids that pyramid resistance genes from less cross-compatible species (Fávero *et al.*, 2020). Importantly, *A. ipaënsis* itself has shown resistance to *Thecaphora frezii* (de Blas *et al.*, 2021; Rosso *et al.*, 2025), trait also observed in diploid hybrids derived from resistant wild species (García *et al.*, 2021), supporting the inheritance of smut resistance traits at the diploid level (Rosso *et al.*, 2025). These findings reinforce the value of *A. ipaënsis* as a genetic donor for the development of resistant pre-breeding materials.

Despite their potential, amphidiploids of interest are currently unavailable in Argentine germplasm collections, limiting their application in local breeding programs. The development of a genetically confirmed amphidiploid through local production would represent a critical step toward expanding the pre-breeding toolbox in Argentina. Such a resource would facilitate the creation of introgressed populations targeting biotic and abiotic resistance and accelerate the development of resistant cultivars adapted to

regional production systems. To address this need, we report here the production and characterization of synthetic amphidiploids derived from previously obtained *A. ipaënsis* × *A. duranensis* diploid hybrids. Two colchicine-based chromosome doubling protocols were tested. The resulting plants were evaluated for ploidy level, heterochromatin banding pattern, pollen viability, and morphological traits. Our objective was to develop a fertile amphidiploid, genetically compatible with *A. hypogaea*, to be incorporated into Argentine germplasm collections as a valuable resource for breeding and for broadening the genetic base of the cultigen throughout national improvement programs.

MATERIALS AND METHODS

Plant material

The diploid hybrid material used was obtained from interspecific crosses previously carried out by our group between *Arachis ipaënsis* KGBPScS 30076 (female) and *A. duranensis* VEVNv 14167 (male), as described in García *et al.* (2020). These accessions correspond to those used to construct the diploid reference genomes of peanut (Bertioli *et al.*, 2016) and are conserved at the Tropical and Subtropical Species Germplasm Bank (BGCTES) of the Instituto de Botánica del Nordeste (IBONE), Corrientes, Argentina. The diploid hybrids were maintained under greenhouse conditions at IBONE until their use for amphidiploid induction. Information of the parental species, diploid hybrids, and induced amphidiploids is provided in Table 1.

Table 1. Taxonomic identity, ploidy level, genome type, and collection data of the parental species, diploid hybrid, and synthetic amphidiploid used in this study. Collectors: B, D. Banks; Ev, A. Echeverry; G, W.C. Gregory; K, A. Krapovickas; Nv, L. Novara; P, J. Pietrarelli; S, C.E. Simpson; Sc, A. Schinini; V, J.F.M Valls. ^a García *et al.* (2020).

Taxon	Ploidy	Genome type	Procedence
<i>A. duranensis</i> Krapov. & W.C. Greg.	2x	AA	Argentina, Salta, Capital Department. 24° 45' S, 65° 26' W. VEVNv 14167.
<i>A. ipaënsis</i> Krapov. & W.C. Greg	2x	BB	Bolivia, Tarija Department, Gran Chaco, Ipa. 21° 00' S; 63° 24' W. KGBPScS 30076.
[<i>A. ipaënsis</i> KGBPScS 30076 × <i>A. duranensis</i> VEVNv 14167] ^{2x}	2x	BA	Induced diploid hybrid (IpaDur ^{2x}) ^a
[<i>A. ipaënsis</i> KGBPScS 30076 × <i>A. duranensis</i> VEVNv 14167] ^{4x}	4x	BBAA	Induced allotetraploid at generation F ₀ (IpaDur ^{4x} F ₀)
[<i>A. ipaënsis</i> KGBPScS 30076 × <i>A. duranensis</i> VEVNv 14167] ^{4x}	4x	BBAA	Induced allotetraploid at generation F ₁ (IpaDur ^{4x} F ₁)

Induction of amphidiploidy via colchicine treatment

Two colchicine-based protocols were tested for inducing chromosome duplication in vegetative cuttings of diploid hybrids:

-Protocol I: The methodology described by Fávero *et al.* (2006) was followed. Cuttings of approximately 20 cm in length were obtained from lateral branches of the diploid hybrids. They were partially immersed (approximately 8-10 cm) in test tubes containing a 0.2 % (w/v) colchicine solution for 8 hours under controlled light and temperature conditions (Fig. 1A).

-Protocol II: A modified procedure based on Santos (2012) was employed. In this case, the entire cuttings were immersed in a 0.2 % (w/v) colchicine solution for 8 hours under controlled light and temperature conditions (Fig. 1B).

After treatment, the cuttings from both protocols were washed under running water for 20 minutes. To promote rooting, the basal ends were immersed in a solution of indole-3-butyric acid (IBA) for 5 minutes (Fig. 1C). Finally, the cuttings were placed in plastic cups containing substrate and were covered with plastic bags to prevent desiccation (Fig. 1D). Rooting was monitored, and rooted cuttings (typically within 15 days) were transplanted into pots and transferred to the greenhouse for further development (Fig. 1E-F).

Flow Cytometry Estimation of Ploidy Level

The ploidy level was estimated by measuring the relative nuclear DNA content using flow cytometry in 40 plants, following the sample preparation protocol recommended by the Partec CyStain UV Precise P kit. For each individual, approximately 0.5 cm² of fresh leaf tissue was co-chopped in a petri dish with a similar amount of leaf material from an internal standard—the induced diploid hybrid IpaDur^{2x}—whose chromosome number had been previously confirmed through root tip counts (García *et al.*, 2020). After adding 0.5 mL of extraction buffer, the tissue was finely chopped with a sharp razor blade. The mixture was incubated for 2 minutes and filtered through a 50 µm nylon mesh into a sample tube containing 1.5 mL of DAPI staining solution (CyStain UV Precise P, Partec). After an additional 2-minute incubation in the dark on ice, stained nuclei were analyzed using a Partec PA II flow cytometer (Partec

GmbH, Münster, Germany). Fluorescence intensity was measured on a linear scale with the Partec FloMax software, with a minimum of 3000 nuclei per sample and a coefficient of variation (CV) ≤ 8 %. Ploidy levels were determined by comparing the fluorescence peaks of each sample to those of the internal standard, and a two-fold increase in fluorescence intensity was used to indicate tetraploidy.

DAPI Staining

All induced allotetraploid plants obtained (IpaDur^{4x}F₀) were analyzed to confirm chromosome duplication and to characterize genome composition. Fluorescent DAPI staining was used to visualize heterochromatic banding patterns distinctive of A and B genome chromosomes: A genome chromosomes (e.g. *A. duranensis*) exhibit prominent centromeric DAPI⁺ bands, while B-genome chromosomes (e.g. *A. ipaënsis*) typically lack these signals (Robledo *et al.*, 2009; Robledo & Seijo, 2010). Root tips obtained from detached-leaf rooting method (Fávero *et al.*, 2005) were treated with 2 mM 8-hydroxyquinoline and subsequently fixed in absolute ethanol/glacial acetic acid solution (3:1) for 12 h at 4 °C (Fernández & Krapovickas, 1994, modified). The root apices were digested in 1 % (w/v) cellulase (from *Trichoderma viridae*; Onozuka R-10, Serva) plus 10 % (v/v) pectinase (from *Aspergillus niger*, Sigma) dissolved in 40 % glycerol in 0.01 M citrate buffer (pH 4.8) for 2 h at 37 °C before squashing, and slides were air dried. The DAPI banding was performed according to the protocol described by Schweizer (1976).

Pollen Viability

Pollen viability was estimated by staining mature pollen grains with carmine:glycerine solution (1:1) (Pittenger & Frolik, 1951). For each hybrid individual, three flower buds were collected and analyzed using the CellCounter plugin from Fiji software (Schindelin *et al.*, 2012). All pollen grains were counted, and those exhibiting regular morphology and intense staining were considered viable. Pollen viability was expressed as the percentage of viable pollen grains over the total observed.

Morphological characterization

Mature fruits were collected from *A. ipaënsis*, *A. duranensis*, their diploid hybrid (IpaDur^{2x}), and synthetic amphidiploids (IpaDur^{4x}F₀ and F₁).



Fig. 1. Chromosome duplication procedures applied to diploid hybrids IpaDur^{2x}. **A:** Cuttings partially immersed in a 0.2 % (w/v) colchicine solution. **B:** Entire cuttings immersed in a 0.2% (w/v) colchicine solution. **C:** Rooting promotion in indole-3-butyric acid (IBA). **D:** Treated cuttings placed in plastic cups with substrate and covered with plastic bags. **E:** Rooted cuttings transferred to pots. **F:** Cuttings maintained in a greenhouse.

Representative samples were photographed under standardized lighting and scale conditions using a digital camera. A total of 25 fruits were analyzed for *A. duranensis* and *A. ipaënsis*, and all ten available fruits were examined for the diploid hybrid. Regarding the synthetic tetraploids, two and 24 fruits were analyzed for IpaDur^{4x} F₀ and IpaDur^{4x} F₁, respectively. Fruit length and width were measured using Fiji software (Schindelin *et al.*, 2012). Calibration was performed using the included scale bar, and measurements were expressed in millimeters. Pericarp texture was assessed visually

and classified qualitatively (reticulated, smooth, or intermediate).

To evaluate whether fruit traits present differences between wild species and the synthetic tetraploids, a statistical analysis was conducted. The diploid hybrid and the F₀ amphidiploid were excluded due to limited sample size. Data distribution was assessed and according to the normality of data, comparisons were performed using either one-way ANOVA or the non-parametric Kruskal–Wallis test. All statistical analyses were conducted in R v.4.3.1 (R Core Team, 2023).

RESULTS AND DISCUSSION

Colchicine treatment

The chromosome doubling protocols tested showed contrasting outcomes. In Protocol I, only 26 % of the 140 treated cuttings survived in the greenhouse and developed new leaves and branches. However, none of these plants produced flowers, and no pegs were observed. Flow cytometry analysis confirmed that all surviving individuals remained diploid. Contrastingly, in Protocol II, 80 % of the 53 treated cuttings successfully developed into established plants (Fig. 2A). Flower buds appeared approximately three months after their transfer to the greenhouse (Fig. 2B). Gynophores were detected in seven individuals five months after planting, and two mature fruits were harvested from one of them three months later (Fig. 2C-D). Both fruits, each of which contained one fully developed seed, were subsequently sown under greenhouse conditions. Germination was successful (Fig. 2E) and the resulting seedlings developed into two mature plants. Flow cytometry analysis revealed genome duplication in both plants. Thus, the polyploid induction efficiency of Protocol II was 3.8 % (2 tetraploids obtained from 53 treated cuttings).

The results of the two colchicine-mediated chromosome duplication protocols indicate that full immersion in colchicine enhances uptake and increases the efficiency of polyploid induction in *Arachis* hybrids. This is consistent with previous findings in *Arachis* (Santos, 2012) and was proposed to be related to the broader surface area of exposed tissues, particularly meristematic regions, which increases the number of actively dividing cells susceptible to colchicine's antimitotic action (Santos, 2012). In general, the colchicine chromosome duplication protocols historically used in *Arachis* are similar, but with minor variations according to the user. Nevertheless, reported success rates in obtaining synthetic amphidiploids using this technique vary considerably (Fávero *et al.*, 2006, 2015a; Mallikarjuna *et al.*, 2011; Leal-Bertioli *et al.*, 2015, 2017; de Paula *et al.*, 2017; Gao *et al.*, 2021; Chu *et al.*, 2021; García & Seijo, 2024). The inclusion of Protocol I in this study was motivated by its long-standing and widespread use in *Arachis*, as most reports of colchicine-mediated chromosome duplication rely on partial

immersion of plant material in the colchicine solution. While this approach has been successful in inducing tetraploidy (Fávero *et al.*, 2006, 2015a; Mallikarjuna *et al.*, 2011; de Paula *et al.*, 2017; Bertioli *et al.*, 2021) our previous attempts using partial immersion did not result in the generation of tetraploid plants. This prompted the exploration of alternative procedures. Consequently, we evaluated both protocols in parallel to compare their performance under identical experimental conditions, and to reassess the effectiveness of the historically used method. Our results further confirm that full immersion in colchicine solution, despite concerns about potential inhibition of root formation (Fávero, 2004), can result in successful rooting and plant establishment under controlled conditions (Santos, 2012). This approach may therefore represent a reliable alternative to partial treatments, especially in cases where standard protocols yield suboptimal polyploidization rates.

Cytogenetic characterization: Chromosome counting through DAPI staining and pollen viability

Cytogenetic analysis using DAPI banding confirmed that both individuals derived plants from Protocol II possessed a tetraploid complement ($2n = 4x = 40$), comprising 20 chromosomes with centromeric DAPI⁺ bands and 20 lacking such signals. Additionally, the small A chromosome pair from A subgenome (A09) and two satellite-bearing pairs (A10 and B10) were identified (Fig. 3A). These results provide clear cytological evidence successful chromosomal duplication of the AB genomic complement originally present in the sterile diploid hybrid (Fig. 3B), resulting in the incorporation of both parental genomes in the synthetic amphidiploids.

The presence of 20 chromosomes with centromeric DAPI⁺ bands (characteristic of the A genome of *A. duranensis*) and 20 chromosomes lacking such bands (corresponding to the B genome of *A. ipaënsis*), aligns with previous descriptions of natural AABB and synthetic BBAA tetraploids (Seijo *et al.*, 2004, 2018; do Nascimento *et al.*, 2018, 2020; Leal-Bertioli *et al.*, 2021). These contrasting heterochromatic patterns serve as robust cytological markers for genome discrimination in *Arachis* (Robledo *et al.*, 2009; Robledo & Seijo, 2010), facilitating the validation of hybrid and polyploid constitution. Notably, the number



Fig. 2. Results obtained from colchicine treatment. **A:** Established plants that developed from cuttings. **B:** Flower buds. **C:** Gynophores. **D:** Fruit. **E:** Plant germinated from seed obtained in the treated cuttings.

and distribution of the centromeric bands in the synthetic amphidiploids resembled those of its diploid progenitors, consistent with a preserved organization of the chromosomal complements after polyploidization. This additive pattern highlights the stability of the heterochromatic fraction during allopolyploidization (Seijo *et al.*, 2018). In addition, the detection of SAT chromosomes A10 and B10, along with the A09 pair, further confirmed the expected BBAA genomic composition.

Pollen viability analysis revealed notably high values in both adult plants, reaching 90.09 % and 93.06 %, respectively (Fig. 3C), contrasting with the sterility observed in diploid hybrids [*A. duranensis* × *A. ipaënsis*]^{2x}, where values typically fall below 5 % (García *et al.*, 2020; Fig. 3D). The restoration of pollen viability in these amphidiploids is consistent with results obtained in previous work using the same species (Fávero *et al.*, 2006; Simpson, 2024),

as well as having been confirmed in amphidiploids derived from hybrids among other wild species (Mallikarjuna *et al.*, 2011; Fávero *et al.*, 2015a, b; Bertoli *et al.*, 2021; Chu *et al.*, 2021; Gao *et al.*, 2021). This sharp increase in viability may indicate the restoration of meiotic functionality, extensively reported to be impaired in intergenomic diploid hybrids (reviewed in García *et al.*, 2024).

Hybrid sterility frequently arises from irregular chromosome pairing during meiosis due to gene incompatibilities or chromosome rearrangements and constitutes a common mechanism of postzygotic reproductive isolation in plants (Dobzhansky, 1941; Grant, 1971). Chromosome doubling, by reconstituting a diploid-like chromosomal balance, is widely recognized as a fundamental strategy to restore meiotic regularity and overcome sterility (Ramanna & Jacobsen, 2003) and has been validated across diverse taxa (Nimura *et*

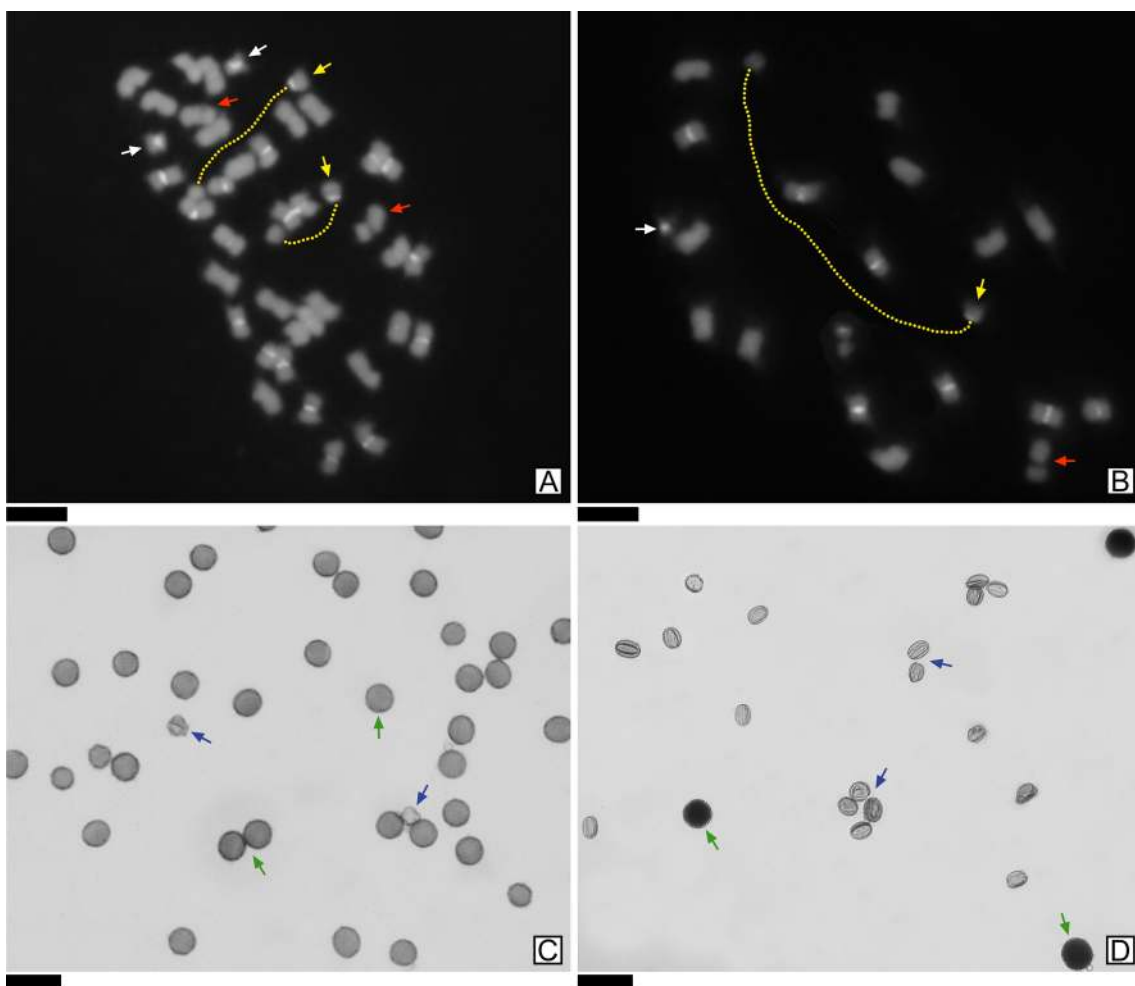


Fig. 3. Cytogenetic comparisons between tetraploid and diploid plants. **A:** DAPI banding pattern in amphidiploids IpaDur^{4x} F₀. **B:** DAPI banding pattern in IpaDur^{2x}. White arrows indicate “A chromosomes,” yellow arrows indicate the satellites in A genome chromosomes, and red arrows indicate the satellites in B genome chromosomes. **C:** Pollen viability in amphidiploid IpaDur^{4x} F₀. **D:** Pollen viability in IpaDur^{2x}. Green arrows viable pollen grains and blue arrows indicate unviable pollen grains. Scale bars= A-B: 5 µm; C-D: 40 µm.

al., 2006; Tel-Zur *et al.*, 2020). In our study, fruit development alongside high pollen viability values, confirmed restored fertility, an essential trait for their utility as bridging materials in breeding. The fertility observed is consistent with previous results in *Arachis* amphidiploid (Simpson, 2024) and reinforces the value of chromosome doubling in restoring reproductive competence in synthetic lines. Additionally, polyploidization has been linked to increased adaptability and genomic flexibility (Islam *et al.*, 2022), suggesting that,

beyond restoring fertility, this process may confer further advantages relevant to crop improvement.

Morphological characterization

The two amphidiploid IpaDur^{4x} F₀ individuals exhibited greater overall robustness, a common feature of polyploid compared to those typically observed in diploids (Fig. 4A-B). This enhanced vigor was evident in several vegetative traits, including increased leaflet and reproductive size, stem thickness, and overall plant vigor,



Fig. 4. Morphological characters of synthetic amphidiploids compared with diploid hybrids and parental species. **A:** Plant of the amphidiploid IpaDur^{4x} F₀. **B:** Plant of the diploid hybrid IpaDur^{2x}. **C:** Flowers of parental species (*A. ipaënsis* and *A. duranensis*, left and right, respectively) and of the amphidiploid IpaDur^{4x} F₀ (center). **D:** Flower of the diploid hybrid IpaDur^{2x}. **E:** Fruit of the diploid hybrid IpaDur^{2x}. **F:** Fruit of the amphidiploid IpaDur^{4x} F₀. Scale bars= A-B: 10 µm; C, E-F: 1 cm; D: approx. 1 cm.

observations that are consistent with previous reports of allotetraploidization effects in *Arachis* (Leal-Bertioli *et al.*, 2017; Levinson *et al.*, 2021).

The flowers produced by the amphidiploid plants were uniformly yellow, including both the standard and wing petals (Fig. 4C). This coloration is consistent with that of the male parent *A. duranensis* (VEvNv 14167) and was also observed by Fávero *et al.* (2006) in the amphidiploid derived from the same parental accessions. Conversely, Simpson (2024) reported an amphidiploid derived from a cross between *A. duranensis* (accession K30067, female parent with orange flowers) and *A. ipaënsis* (male parent), in which the resulting plants displayed orange flowers. Although Krapovickas and Gregory (1994) described *A. ipaënsis* as having yellow flowers, plants grown in the BGCTES greenhouse consistently display an orange hue (Fig. 4C), aligning with Fávero *et al.* (2006)

observations. In contrast, *A. duranensis* exhibits intraspecific variation in flower color, ranging from yellow to orange (Krapovickas & Gregory, 1994). The yellow flower color observed in these amphidiploids is consistent with that of the diploid hybrid used for chromosome duplication (Fig. 4D), as well as with other synthetic amphidiploids. Tetraploid plants derived from hybrids involving wild species that naturally produce yellow flowers, such as *A. batizocoi*, *A. stenosperma*, and *A. duranensis* (Krapovickas & Gregory, 1994), commonly exhibit this trait (Fávero *et al.*, 2006; Mallikarjuna *et al.*, 2011; Bertioli *et al.*, 2021; Chu *et al.*, 2021; Gao *et al.*, 2021). Although it has been proposed that flower color is inherited from the male parent (Leal Bertioli *et al.*, 2015), the consistent appearance of yellow flowers in hybrids derived from yellow-flowered species, even when used as the female parent (unpublished), suggests

that this trait may behave as a dominant character, regardless of parental direction. This pattern is consistent with observations in other legumes, such as *Vigna unguiculata* and *Vicia faba*, where flower pigmentation often follows simple Mendelian inheritance involving one or two major genes (Othman *et al.*, 2006; Hughes *et al.*, 2020). Studies in *Vigna unguiculata* (Sangwan & Lodhi, 1998) reported dominant inheritance of standard color, and flower pigmentation has been proposed as a reliable phenotypic marker in breeding programs. These findings across taxa support the hypothesis that yellow pigmentation in *Arachis* hybrids may also be governed by one or a few dominant alleles. Further genetic studies focused on individual floral structures will be essential to fully understand the inheritance patterns and their potential utility in early selection schemes.

Regarding pericarp texture, the diploid hybrid (IpaDur^{2x}) presented a slightly reticulated pericarp, whereas both amphidiploid generations (IpaDur^{4x} F₀ and F₁) presented a fully reticulated pericarp surface (Fig. 4E-F), consistent with the morphology described for *A. ipaënsis* (Krapovickas and Gregory, 1994). Similar results were reported in other synthetic amphidiploids derived from the same parental combination (Leal-Bertioli *et al.*, 2017; Simpson, 2024), suggesting conservation of this trait following genome doubling.

Fruit morphology analysis revealed differences among the parental species, the diploid hybrid (IpaDur^{2x}), and the different generations of the synthetic amphidiploids (IpaDur^{4x}F₀ and F₁) (Table 2; Fig. 5). While *A. ipaënsis* and *A. duranensis* produced fruits with mean lengths of 10.72 mm and 11.2 mm, respectively, the diploid hybrid

(IpaDur^{2x}) exhibited an increased fruit length of 13.94 mm. Further increments were observed in both generations of amphidiploids, with mean lengths of 14.48 mm in the F₀ and 14.41 mm in the F₁. A similar trend was observed for fruit width. The parental species produced narrower fruits (5.31 mm in *A. ipaënsis* and 4.97 mm in *A. duranensis*), whereas the diploid hybrid and amphidiploids displayed broader fruits, reaching up to 7.09 mm in IpaDur^{4x} F₁.

Statistical analysis of fruit length revealed significant differences among the wild species and the IpaDur^{4x} F₁ (H = 73.26, p < 0.0001). According to post hoc comparisons, the two wild species did not differ significantly from each other, but both differed significantly from the tetraploid group. As for fruit width, the analysis of variance (ANOVA) revealed significant differences among the evaluated individuals (F = 91.9; p < 0.0001). Tukey multiple comparison test (α = 0.05) identified that *A. ipaënsis*, *A. duranensis* and the amphidiploid IpaDur^{4x} F₁ form three significantly different groups. Interestingly, these results contrast with previous findings where no significant variation in pod dimensions was reported between amphidiploids and their wild ancestors (Leal-Bertioli *et al.*, 2017). Furthermore, while Simpson (2024) noted that amphidiploid fruits present phenotypic similarity with those of *A. monticola*, no quantitative comparisons were presented. The consistent fruit enlargement observed in the amphidiploids obtained in this work may result from genomic duplication and/or heterotic effects (Garg *et al.*, 2025). This result represents an early morphological marker of domestication-relevant traits and could serve as a practical character for early selection in pre-breeding programs.

Table 2. Characterization of fruit traits in <i>A. ipaënsis</i> , <i>A. duranensis</i> , the diploid hybrid (IpaDur ^{2x}), and both generations of the synthetic amphidiploids (IpaDur ^{4x} F ₀ and F ₁). Abbreviation= n: sample size.					
Characters	<i>A. ipaënsis</i>	<i>A. duranensis</i>	IpaDur ^{2x}	IpaDur ^{4x} F ₀	IpaDur ^{4x} F ₁
n	25	25	10	2	24
Fruit length range	9.04 - 12.59	10.19 - 12.18	12.08 - 16.41	14.06 - 14.77	13.21 - 15.73
Mean fruit length (mm)	10.76	11.20	13.94	14.48	14.41
Fruit width range	4.92 - 7.00	4.08 - 6.35	5.72 - 8.09	6.16 - 6.62	6.04 - 9.15
Mean fruit width (mm)	5.75	4.98	6.95	6.40	7.09
Pericarp	Reticulated	Smooth	Slightly reticulated	Reticulated	Reticulated

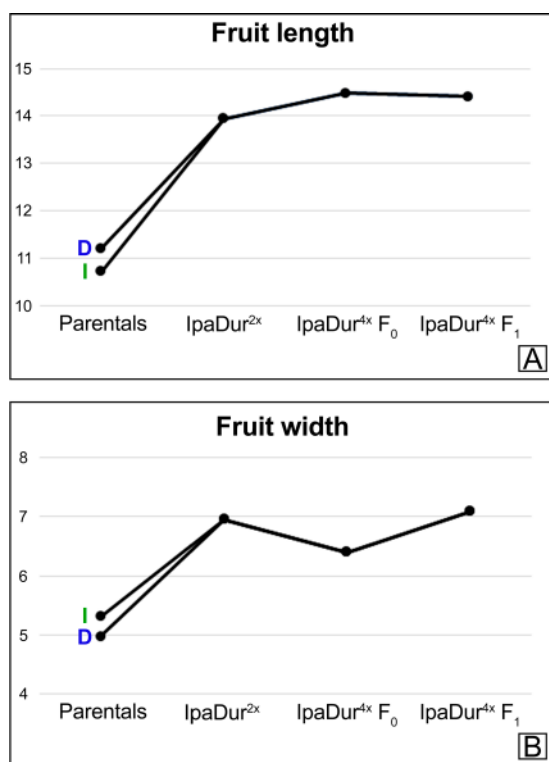


Fig. 5. Fruit traits measured in the parental species *A. ipaënsis* (indicated with a green I) and *A. duranensis* (indicated with a blue D), the diploid hybrid (IpaDur^{2x}), and both generations of the synthetic amphidiploids (IpaDur^{4x} F₀ and F₁). **A:** Fruit length. **B:** Fruit width. Values represent the average fruit measurements in mm for each genotype.

CONCLUSIONS

The generation of a locally produced, fertile, and cytogenetically validated amphidiploid between *A. ipaënsis* and *A. duranensis* represents a critical advance for peanut pre-breeding in Argentina. This amphidiploid effectively fills a long-standing gap in the availability of introgression material, strengthening national capacity to develop cultivars adapted to local agroecological conditions and resistant to threats. Its validation through chromosome counting, high pollen viability, and restored fertility confirms its immediate utility in breeding programs as a reliable and agronomically valuable genetic bridge. While further studies are needed to assess its hybridization behavior with

A. hypogaea and long-term genomic stability, it will also be essential to evaluate its performance under field conditions, particularly its response to *Thecaphora frezii*, a major pathogen threatening peanut production in Argentina. This material thus establishes a strategic platform for both applied improvement and future research on polyploid genome dynamics in *Arachis*, while also providing a valuable experimental system to investigate how interspecific hybridization and chromosome duplication shape phenotypic and cytogenetic trait expression, thereby supporting the development of more predictive and efficient breeding strategies.

AUTHOR CONTRIBUTIONS

#AVG and AMO have contributed equally to this work. AVG: Data curation, formal analysis, investigation, methodology, visualization, writing (original draft), writing (review and editing). AMO: Data curation, formal analysis, investigation, methodology, writing (original draft), writing (review and editing). JA: Data curation and investigation. GIL: Conceptualization, data curation, funding acquisition, project administration, supervision, validation, writing (original draft), writing (review and editing).

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