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REGISTRATION

Germplasm

Registration of six disease resistant, high protein, induced allotetraploids derived from *Arachis duranensis* and *A. ipaënsis*, the genome progenitors of peanut

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Abstract

The allotetraploid legume peanut (*Arachis hypogaea* L.) has low genetic diversity due to the single origin and ploidy barrier with its wild relatives, which are mostly diploid. This lack of genetic diversity hinders breeding and genetic gains. Wild peanut relatives harbor known resistances to pests and diseases and genetic variation that can contribute to adaptation to diverse environments and a changing climate. The ploidy barrier between the wild relatives and the cultigen can be surpassed by the production of induced allotetraploids using parents with genomes that are compatible to those of peanut. Here, we crossed seven accessions of six *Arachis* species. All combinations carried at least one of the peanut progenitors, *A. ipaënsis* and *A. duranensis*, crossed with a species of complementary genome. The original hybrids were treated with colchicine for recovery of fertility, observed by pollen viability and seed production. Six new allotetraploids were produced; they were named WPL-BatDur1 (Reg. no. GP-248, PI 707934), WPL-BatDur2 (Reg. no. GP-249, PI 707936), WPL-MagDur1 (Reg. no. GP-253, PI 707942), WPL-IpaCor1 (Reg. no. GP-251, PI 707938), WPL-IpaDur1 (Reg. no. GP-250, PI 707937), and WPL-IpaVillo1 (Reg. no. GP-252, PI 707941), released by the University of Georgia. Cytogenetic analyses confirmed the tetraploid plants had 20 A-type and 20 B- or K-type chromosomes. All these

Abbreviations: DAP, days after planting; DAPI, 4,6-diamidino-2-phenylindole; ELS, early leaf spot; LLS, late leaf spot.

allotetraploids have surprisingly high protein content. They also show moderate to strong resistance to the main peanut fungal diseases (late and early leaf spot and rust) and Southern stem rot, are compatible with cultivated peanut, and are being used in breeding programs in the United States, Senegal, and Brazil for the development of resilient peanut cultivars.

Plain Language Summary

Peanuts are vulnerable to many pests and diseases, and because they have low resistance, they often require extensive pesticide use. In nature, wild relatives of peanuts are resistant to these threats, but they can't be directly crossed with cultivated peanuts due to genetic incompatibility. To overcome this, we developed six hybrids, called here "induced allotetraploids" using the wild relatives. These hybrids not only have strong resistance, but also high protein content and can successfully be crossed with peanuts. This allows us to introduce resistance genes into peanut crops, improving their resilience and reducing pesticide use. This is an important step toward making peanut farming more sustainable and cost-effective.

1 | INTRODUCTION

Peanut (*Arachis hypogaea* L.) also known as groundnut, holds a significant position as one of the most widely grown legume crops worldwide. Cultivated extensively across the tropics and sub-tropics, it plays a crucial role as a staple food in many regions, serving as a primary protein and oil source in various countries and is also important as animal feed (Settaluri et al., 2012). Peanut is an allotetraploid species ($2n = 4x = 40$) that evolved from a cross between the two diploid species *A. duranensis* Krapov. and W.C. Gregory (female, A-genome species) and *A. ipaënsis* Krapov. and W.C. Gregory (male, B-genome species) and spontaneous tetraploidization. This happened in the region between the northeast of Argentina and south of Bolivia around 8000 years ago (Bertioli et al., 2016; 2019). After its origin, between 10,000 and 5000 years ago, the new allotetraploid species was domesticated in complete genetic isolation from the diploid wild relatives, generating low genetic diversity in the peanut cultigen (Kochert et al., 1996). Moreover, genetic variability in elite cultivars grown in the United States suffered a recent, secondary bottleneck, as they are derived from very few plant introductions and are genetically related (Otyama et al., 2020). This restricts the potential for genetic enhancement through crosses involving cultivated germplasm.

Eighty-four formally recognized species have been described by Krapovickas and Gregory (1994, 2007), Santana and Valls (2015), Seijo et al. (2021, 2024), Valls et al. (2013), and Valls and Simpson (2005; 2017), and new species are yet to be described. The *Arachis* section to which peanut belongs has 34 species, 32 of which are diploid and therefore not readily crossable with peanut. An effective route for introgressing genes from the secondary gene pool into the cultigen is the cross of diploid species with complementary genomes and tetraploidizing the resulting sterile hybrid. This is called the tetraploid route and was originally described by Simpson (1991). This was used to create the first wild-derived root-knot nematode resistant lines and cultivars (Simpson & Starr, 2001; Simpson et al., 1993). This route was later simplified by Fávero et al. (2006) and has been used to create several peanut-compatible allotetraploids (Bertioli et al., 2021; Chu et al., 2021; Gao et al., 2021; Leal-Bertioli et al., 2015b; 2017; 2021; Mallikarjuna et al., 2011), making wild alleles readily available for crop breeding. Thus far, the main use of wild species alleles has been for pest and disease resistance, because peanut has only moderate levels of resistance to these diseases (Holbrook & Stalker, 2003).

Here we describe the creation of six new induced allotetraploids (also called neotetraploids) derived from crosses using as parents, *A. ipaënsis or A. duranensis*, the diploid species that were the original paternal and maternal progenitor of peanut, respectively. These species were used to make crosses with *A. batizocoi*, *A. magna*, *A. correntina*, and *A. villosa*. These neotetraploids are compatible with cultivated peanut, show resistance to Late (LLS) and Early Leaf Spots (ELS) (caused by the fungi *Northopassalora personata* and *Passalora arachidicola*, respectively), rust (caused by *Puccinia arachidis*) and Southern stem rot (caused by *Agroathelia rolfsii*) and are being used in breeding programs. They were named WPL-BatDur1 (Reg. no. GP-248, PI 707934), WPL-BatDur2 (Reg. no. GP-249, PI 707936), WPL-MagDur1 (Reg. no. GP-253, PI 707942), WPL-IpaCor1

(Reg. no. GP-251, PI 707938), WPL-IpaDur1 (Reg. no. GP-250, PI 707937), and WPL-IpaVillo1 (Reg. no. GP-252, PI 707941). WPL stands for UGA-Wild Peanut Lab, where the crosses were done.

2 | METHODS

2.1 | Pedigree and history

All allotetraploids originate from crosses between accessions of the two species of the progenitors of peanut. Three allotetraploids have as female parent, the only accession available of *A. ipaënsis* (GKBSPSc 30076 PI 468322). This accession was crossed with an A-genome species: *A. correntina*, *A. duranensis*, or *A. villos*a. The other three allotetraploids have, as male parent, an accession of *A. duranensis* that was crossed with a B- or K-genome species (*A. magna* or *A. batizocoi*). These are all diploid peanut wild relatives and possess resistances to various peanut pests and diseases, including rust (caused by *Puccinia arachidis*), scab (caused by *Sphaceloma arachidis*), thrips (caused by *Frankliniella fusca*), and fall armyworm (caused by *Spodoptera frugiperda*) (Table 1). The accessions come from a wide range of environments across Argentina, Bolivia, and Uruguay, over a range of over 1600 km (1000 mi), and are all deposited at the National Plant Germplasm System (NPGS) (Table 1, Figure 1). Full information on these accessions, including their place and climatic conditions of origin can be found at https://www.genesys-pgr.org.

2.2 | Allotetraploid production

The original seeds for the crosses were obtained from the USDA collection (http://www.ars-grin.gov/). Crosses were made in greenhouse and generated sterile diploid hybrids. Cuttings from the diploid hybrids were treated with 0.2% colchicine overnight as previously described (Leal-Bertioli et al., 2015b). Cuttings were maintained for several months in sand supplemented with liquid NPK (10:10:10), after which, a few germinated allotetraploid plants were observed. Confirmation of tetraploidization was by pollen fertility, chromosome count and DAPI^{+} (4′, 6-diamino-2-phenylindole) staining (See "Cytogenetics" section). Pollen fertility was measured by staining pollen grains of four flowers with 2% acetic carmine (Alexander, 1969).

The composition of the induced allotetraploids is as follows:

WPL-IpaCor1: [*A. ipaënsis* GKBSPSc 30076 PI 468322 x *A. correntina* GKP 9548 PI 262881]^{4x}.

WPL-IpaDur1: [*A. ipaënsis* GKBSPSc 30076 PI 468322 x *A. duranensis* V 14167 PI 692197]^{4x}.

TABLE 1 Original diploid Arachis accessions used for crosses.

Species	Genome type	Plant ID		Collection site	Coordinates (lat, long)	Resistance[a]	References[b]
		Collector's code	PI no.				
A. batizocoi	KK	K 9484	PI 298639	Santa Cruz, Bolivia	−20.01, −63.32	Rust, RKN, ELS, LLS, scab, thrips	L1, B, F, M1, M2
A. correntina	AA	GKP 9548	PI 262881	San Cosme, Argentina	−27.74, −58.83	Rust, ELS, and LLS	L1, this study
A. duranensis	AA	VNvEc 14167	PI 692197	Salta, Argentina	−24.77, −65.45	Rust, ELS, LLS and scab	L1, F, M1
A. ipaënsis	BB	GKBSPSc 30076	PI 468322	Machareti, Bolivia	−21.1, −63.34	FA, thrips, Rust, ELS, LLS	F, L1, L2, M1, M2
A. magna	BB	KGSSc 30097	PI 468340	Santa Cruz, Bolivia	−16.4, −63.40	Rust, ELS, LLS, scab	L1, LB, M1
A. villosa	AA	VGoMrOvGv 12812	PI 330651	Bella Unión, Uruguay	−30.03, −57.76	Rust, ELS, and LLS	L1, F

[a]Rust, caused by *Puccinia arachidis*; ELS, early leaf spot caused by *Passalora arachidicola*; LLS, late leaf spot, caused by *Northopassalora personata*; scab, caused by *Sphaceloma arachidis*; FA, Fall armyworm; *Spodoptera frugiperda*; thrips, *Enneothrips flavens*.

[b]References for resistance of the accessions: L1, Levinson et al. (2021); B, Ballén-Taborda et al. (2019); F, Fávero et al. (2009); M1, Michelotto et al. (2015); L2, Levinson et al. (2020); M2, Michelotto et al. (2017), LB, Leal-Bertioli et al. (2015a).

FIGURE 1 Geographical site of collection of the accessions used for allotetraploid production: *A. magna* GKSSc 30097 (PI 468340), *A. batizocoi* K9484 (PI 298639), *A. ipaënsis* K 30076 (PI 468322), *A. duranensis* V 14167 (PI 692197), *A. correntina* GKP 9548 (PI 262881), and *A. villosa* V 12812 (PI 330651). Note that A-genome species are represented with a square and non-A genome species are represented with a circle. Coordinates were plotted using GoogleMyMaps.

WPL-IpaVillo1: [*A. ipaënsis* GKBSPSc 30076 PI 468322 x *A. villos*a V 12812 PI 330651]^{4x}.

WPL-MagDur1: [*A. magna* GKSSc 30097 PI 468340 x *A. duranensis* 14167 PI 692197]^{4x}.

WPL-BatDur1: [*A. batizocoi* K9484 PI 298639 x *A. duranensis* V14167 PI 692197]^{4x}.

WPL-BatDur2: [*A. batizocoi* K9484 PI 298639 x *A. duranensis* KSSc 38906 PI 497270]^{4x}.

2.3 | Cytogenetics

In order to analyze chromosome number and genome composition of the allotetraploids, chromosome counts and DAPI staining were done essentially as described in Nascimento et al. (2018). In short, metaphase spreads were obtained from root tips collected from young plants, treated with 8-hydroxyquinoline, fixed in ethanol/glacial acetic acid (3:1, v/v) and digested with proteolytic enzymes (cellulase 2% and pectinase 20%). Chromosomes were spread in a drop of 60% acetic acid on a slide. Subsequently slides were treated with DAPI^{+} (4′, 6-diamino-2-phenylindole) diluted in McIlvaine buffer (citrate-phosphate buffer) (2 µg/mL). Slides were mounted using a glycerol/McIlvaine buffer (1:1, v/v) solution added to 2.5 mM $MgCl_2$ in dark conditions at room temperature. Observation was done in Zeiss AxioPhot microscope using UV light and excitation/emission fluorescence filters.

2.4 | Disease resistance

Allotetraploids were tested in vitro for ELS and LLS using methodologies previously described in (Leal-Bertioli et al., 2015a; Moraes & Salgado, 1982). The response of allotetraploids to stem rot were also tested using the methodology of Tsai et al. (2023). The measured lesion lengths of stem rot were subject to analysis of variance by using non-parametric Kruskal–Wallis test (RStudio version 1.2.1335) to examine the significant differences among treatments. Dunn test was then calculated for mean separations among treatments for each evaluation method (RStudio version 1.2.1335).

2.5 | Seed quality

Allotetraploid plants were grown in a screen house in 2023 at the University of Georgia, Griffin campus using standard cultivation practices. Three replicates were grown for

each allotetraploid as well as cultivated controls. Fresh harvested seeds were measured for seed traits including total oil, total protein, and oleic acid percentage. Oil was measured by bulk seed analysis using nuclear magnetic resonance (NMR) on a Bruker minispec mq-one Seed Analyzer (Wang et al., 2022). For protein content, individual seeds were crushed and measured by combustion on an Elementar Rapid N Exceed nitrogen analyzer. Nitrogen values were converted to protein percentage using the protein factor 5.46 for peanuts (Mariotti et al., 2008; Wang et al., 2016). Oleic acid content was also measured as this fatty acid is an important component influencing the stability of oil. It was calculated as a percentage of the total fatty acids by converting an oil sample to fatty acid methyl esters (FAMES) and analyzing on an Agilent 7890A gas chromatograph with a flame ionization detector (FID) (Wang et al., 2013). Experiments were repeated in the 2024 season. Measurements for each seed trait were subjected to analysis of variance in R Studio. Phenotypic data were statistically analyzed using the softwareR (R Core Team, 2022). Parametric test assumptions were checked and verified using the Shapiro–Wilk and Breusch–Pagan tests. Average comparisons were done using standard variance analyses, followed by the Tukey HSD test at 5% significance levels.

2.6 | Genotyping markers

In order to identify hybrids between the allotetraploids and cultivated peanut, single nucleotide polymorphism (SNP) markers that distinguished between the allotetraploids and a panel of 384 *A. hypogaea* accessions from all six botanical varieties were selected. All accessions were genotyped using the Axiom Arachis v02 (ThermoFisher) (Clevenger et al., 2017; Korani et al., 2019; Pandey et al., 2017). To identify informative assays, we aimed to identify SNPs that differentiated each accession in each combination. Therefore, markers specific to any particular allotetraploid are not in any of the 384 *A. hypogaea* accessions genotyped.

3 | CHARACTERISTICS

3.1 | General plant traits

All allotetraploids evaluated were fertile and compatible with peanut. Hybrids between these and *A. hypogaea* subsp. *hypogaea* give a consistent viable pollen count of over 68%. Seeds have strong dormancy which can be broken by treating them overnight with Ethephon 0.05%. In front of each germplasm name is the Plant Introduction number, assigned by the NPGS, as well as the link to the page at the USDA-ARS Germplasm Resources Information Network (GRIN) website.

WPL-BatDur1 (PI 707934) has a prostrate growth habit, with alternate branching pattern, purple stem pigment, elliptic main stem leaves, obovate side stem leaves (Figure 2), and pubescence on abaxial leaf surface. The average apical leaflet length was 46.58 mm with a width of 35.75 mm. Basal leaflet average length was 45.31 mm with a 29.76-mm width. Measurements were taken from the first opened leaf from lateral branches. WPL-BatDur1 takes 55 days to anthesis and has yellow flowers and pod with no reticulation and marked beak. Seed dimensions were as follows: L = 13.0 mm, W = 5.9 mm, 100-seed weight = 22.48 g. Under field conditions, main stem height was 34.6 cm 85 days after planting (DAP). It has 72.2% pollen stainability (n = 6500 pollen grains).

WPL-BatDur2 (PI 707936) has a prostrate growth habit with an alternate branching pattern. The stem is purple at base and transitions into green moving apically, elliptic main stem leaves and obovate side stem leaves (Figure 2). WPL-BatDur2 has pubescence on abaxial leaf surface. Apical leaflet average length was 53.62 mm and width was 40.10 mm. Basal leaflet average length was 51.06 mm and width was 31.59 mm. Measurements were taken from the first opened leaf from lateral branches. WPL-BatDur2 takes 55 days to anthesis and has yellow flowers and pod somewhat reticulate with marked beak. Average seed dimensions were as follows: L = 13.6 mm, W = 5.7 mm, 100-seed weight = 18.14 g. Main stem height was not taken. It has 88.2% pollen stainability (n = 5100 pollen grains).

WPL-MagDur1 (PI 707942) has a prostrate growth habit with alternate branching pattern, green stem pigment, elliptic main stem leaves, and obovate side stem leaves (Figure 2). WPL-MagDur1 has pubescence on abaxial leaf surface. The average apical leaflet length was 53.7 mm and width was 30.01 mm. The average basal leaflet length was 52.71 mm and width was 24.62 mm. Measurements were taken from the first opened leaf from lateral branches. WPL-MagDur1 takes 55 days to anthesis and has yellow flowers, and pod somewhat reticulate with marked beak. Average seed dimensions were as follows: L = 13.2 mm, W = 6.4 mm, 100 seed weigh t = 22.84 g. Under field conditions, main stem height was 38.2 cm 85 DAP. It has 90.1 pollen stainability (n = 1971 pollen grains).

WPL-IpaCor1 (PI 707938) has a prostrate growth habit with prominent main stem, and alternate branching pattern, purple stem pigment, elliptic main stem leaves, obovate side stem leaves, and pubescence on abaxial and adaxial leaf surfaces. Apical leaflet average length 50.83 mm and width 32.02 mm. The average basal leaflet length was 48.36 mm and width was 24.70 mm. WPL-IpaCor1 takes 55–60 days to anthesis and has orange flowers and pod somewhat reticulate with marked beak. Average seed dimensions were as follows: L = 12.6 mm, W = 5.8 mm, 100-seed weight = 20.55 g. Under field conditions, main stem height was 19.4 cm 85 DAP. It has 94.2% pollen stainability (n = 4773 pollen grains).

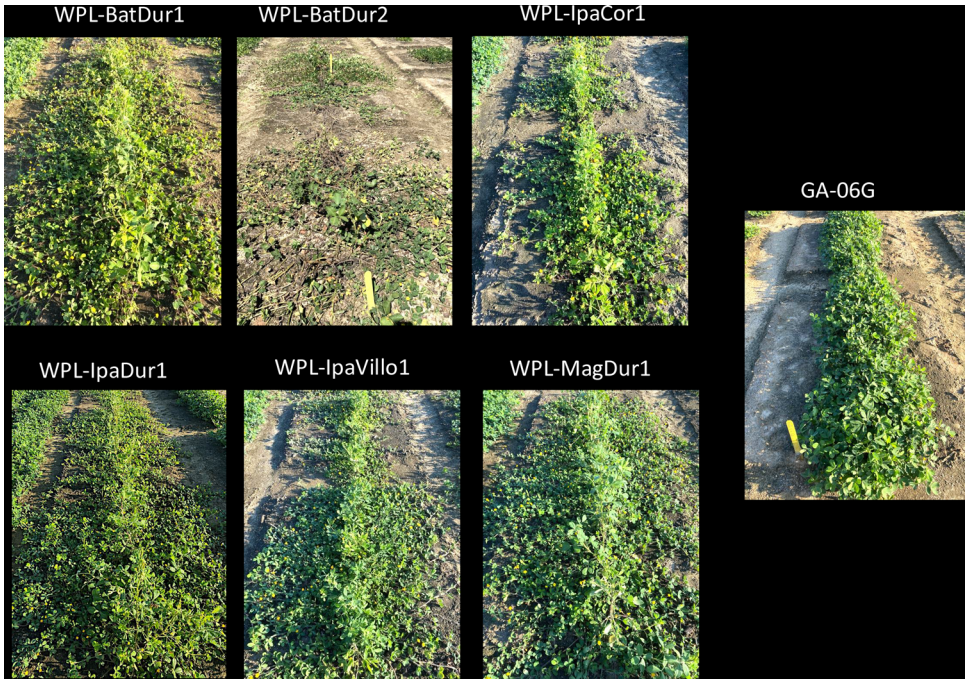

FIGURE 2 Rows of 80-day-old plants of the induced allotetraploids WPL-BatDur, WPL-BatDur2, WPL-IpaCor1, WPL-IpaDur1, WPL-IpaVillo1, WPL-MagDur1, and the cultivated peanut Georgia-06G (GA-06G). Photos were taken at the University of Georgia Southeast Georgia Research and Education Center, in Midville, GA, on August 19, 2021.

WPL-IpaDur1 (PI 707937), an allotetraploid, was created using the species that gave rise to cultivated peanut, and the accessions that have their full genome sequenced (Bertioli et al., 2016). It has prostrate growth habit with prominent main stem (Figure 2) and alternate branching pattern, green stem pigment, elliptic main stem leaves, obovate lateral branch leaves, and pubescence on abaxial leaf surface. The average apical leaflet length was 52.18 mm and width was 32.2 mm. Basal leaflet average length 45.43 mm and 25.13 mm width. WPL-IpaDur1 takes around 62 days to anthesis and has yellow flowers and pod with deep reticulation and marked beak. Seed dimensions were as follows: L = 11.38 mm, W = 6.85 mm, 100-seed weight = 17.3 g. Under field conditions, main stem height was 42.4 cm 85 DAP. It has 84.7% pollen stainability (n = 2431 pollen grains).

WPL-IpaVillo1 (PI 707941) has a prostrate growth habit, with prominent main stem (Figure 2) and alternate branching pattern. Its stem is purple at base and transitions into green moving apically. WPL-IpaVillo1 has elliptic main stem leaves, obovate side stem leaves, and pubescence on abaxial leaf surface. The average apical leaflet length was 51.05 mm and width was 29.44 mm. Average basal leaflet length was 45.38 mm and width was 29.41 mm. WPL-IpaVillo1 takes 62 days to anthesis and has orange flowers and pod somewhat reticulate with marked beak. Average seed dimensions were as follows: L = 12.3 mm, W = 6.2 mm, 100-seed weight = 20.07. Under field conditions, main stem height was 18.3 cm 85 DAP. It has 89.4% pollen stainability (n = 5965 pollen grains).

Growth habit was observed in the field in Midville, GA, in 2021. Main stem height measurements were taken at 80 DAP and under the same conditions. The controls, 'Tifrunner' (Holbrook & Culbreath, 2007) and 'Georgia-06G' (Branch, 2007), had 20.2- and 16.9-cm main stem height, respectively. Leaf measurements were taken from the first opened leaf from lateral branches. For all allotetraploids, growth habit in the field is shown in Figure 2, morphology of side stem leaves is shown in Figure 3 and pod/seed morphology is shown in Figure 4.

3.2 | Cytogenetics

All allotetraploids harbored 40 chromosomes, mostly metacentric, with only two pairs of submetacentric chromosomes (36 m + 4sm). Allotetraploids WPL-IpaCor1, IpaDur1, IpaVillo1, and MagDur1 had 20 chromosomes with DAPI band in centromere region (A-genome) and 20 chromosomes with no detectable DAPI band (B-genome) (Figure 5A, 5B, 5C and 5F). Allotetraploids WPL-BatDur1 and WPL-BatDur2 had 38 DAPI positive chromosomes and only two with no observed DAPI bands, consistent with the *A. batizocoi* K-subgenome (Robledo & Seijo, 2010) (Figure 5D and 5E). These results confirm the tetraploid state and the genome donors of these allotetraploid plants.

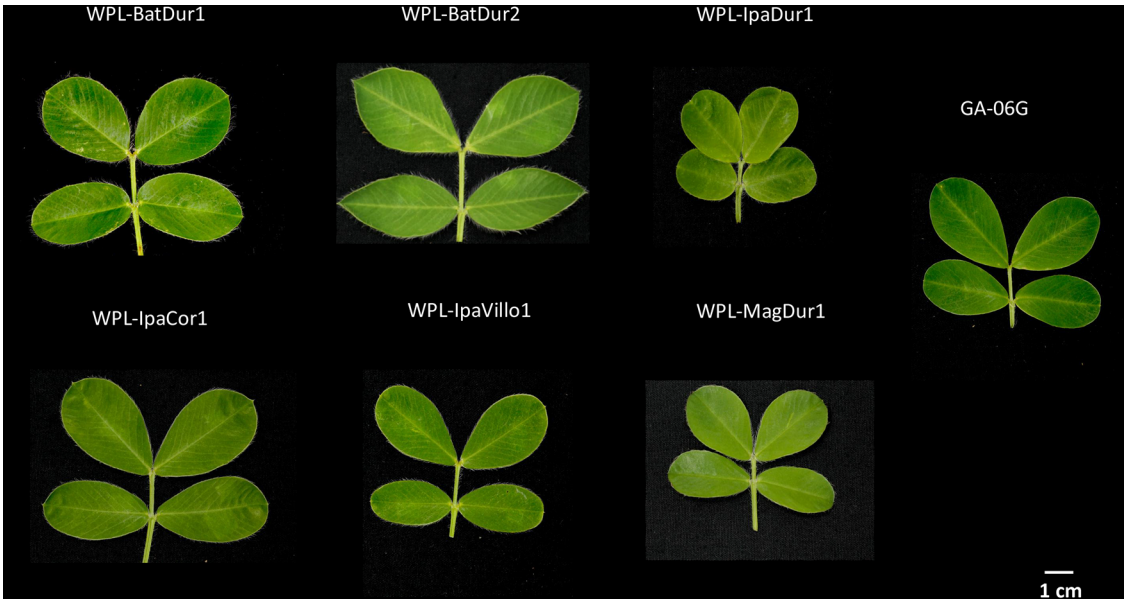

FIGURE 3 Examples of first expanded leaves of induced allotetraploids WPL-BatDur, WPL-BatDur2, WPL-IpaCor1, WPL-IpaDur1, WPL-IpaVillo1, WPL-MagDur1, and the cultivated peanut Georgia-06G (GA-06G). Bar corresponds to 1 cm.

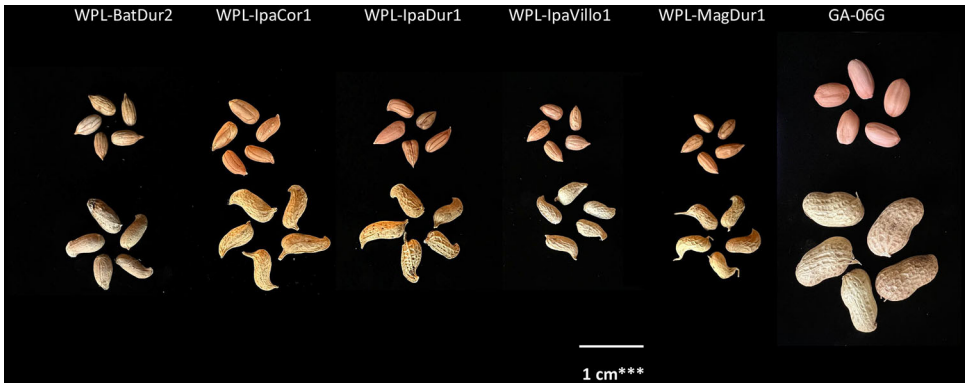

FIGURE 4 Pods and seeds of induced allotetraploids: WPL-BatDur2, WPL-IpaCor1, WPL-IpaDur1, WPL-IpaVillo1, WPL-MagDur1, and the cultivated peanut Georgia-06G (GA-06G). Bar corresponds to 1 cm.

3.3 | Disease resistance

The allotetraploids WPL-BatDur1, WPL-BatDur2, WPL-MagDur1, WPL-IpaCor1, and WPL-IpaVillo1 presented strong resistance to LLS, with lower percentage of diseased leaf area (DLA), lower area under the disease curve (AUDPC) and higher incubation period than the cultivated controls. WPL-IpaCor1 also showed strong ELS resistance. WPL-IpaDur1, derived from the progenitor species of peanut, have similar susceptibility to ELS and LLS as cultivated genotypes (Table 2). All allotetraploids showed partial resistance to stem rot, the disease progressions are slower than susceptible control Georgia-09B from 5 to 7 days after inoculation. All six allotetraploids have numerically lower AUDPC values than Georgia-09B and are comparable to that of 'Georgia-12Y' (Branch, 2013) (Table 3). Although several authors observed that *A. ipaënsis* is highly susceptible to rust (Fávero et al., 2009; Leal-Bertioli et al., 2015a), in a separate study, all six allotetraploids, including the ones that include *A. ipaënsis* as a parent, were found to be significantly more resistant to rust than the cultivated control Georgia Green (Levinson et al., 2021).

3.4 | Seed quality

Three quality parameters were measured in peanut and allotetraploid seeds: oil content, oleic acid, and protein content. No significant differences in oil content were observed; allotetraploid seeds had an average of 45.4% oil content (range

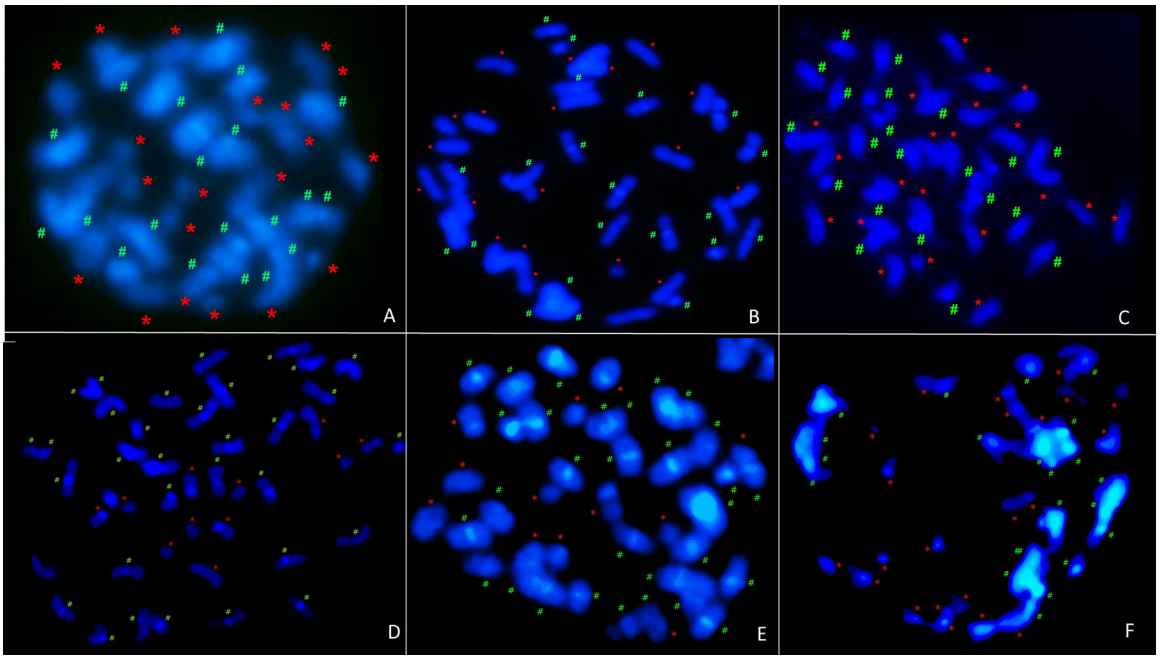

FIGURE 5 Metaphasic chromosomes of the allotetraploids WPL-IpaCor1 (A), WPL-IpaDur1 (B), WPL-IpaVillo1 (C), WPL-BatDur1 (D), WPL-BatDur2 (E), and WPL-MagDur1 (F) displaying 40 chromosomes. In A, B, C, and F, half of the chromosomes show DAPI positive bands in the centromere region (green hashtags), corresponding to the A genome chromosomes while the others lack this band (red asterisks), corresponding to B genome chromosomes. D and E show 38 chromosomes DAPI positive (green hashtags) and only two lack DAPI bands (red asterisks), as the result of *A. batizocoi* (K genome) participation as a parental donor of these centromere DAPI negative chromosomes.

TABLE 2 Results of in vitro evaluation of late leaf spot (LLS, caused by *Nothopassalora personata*) of WPL-IpaDur1, GA-BatDur1, GA-BatDur2, GA-MagDur1, WPL-IpaCor1, and WPL-IpaVillo1. Peanut cultivars Bailey, GA-06G, and TifNV-High O/L were used as controls. Results are expressed as Average ± standard deviation.

	LLS, *Nothopassalora personata*			ELS, *Passalora arachidicola*		
Genotype/trait	**DLA**	**IP**	**AUDPC**	**DLA**	**IP**	**AUDPC**
WPL-IpaDur1	8.04 ± 4.37 ab	15 ± 2.1 ab	4.27 ± 1.29 cd	20.82 ± 8.19 ab	12 ± 1.3 bc	13.54 ± 4.36 abc
WPL-IpaCor1	1.25 ± 0.57 cd	19 ± 3.0 bcd	1.03 ± 0.67 d	1.96 ± 1.06 c	16 ± 2.5 a	4.08 ± 2.44 d
WPL-IpaVillo1	2.37 ± 2.22 cd	18 ± 2.9 bc	1.01 ± 1.09 d	13.21 ± 10.05 abc	13 ± 2.5 abc	10.63 ± 7.89 abcd
WPL-BatDur1	0.60 ± 0.75 d	21 ± 4.5 cd	0.00 ± 0.00 d	10.41 ± 9.81 bc	14 ± 3.1 ab	4.22 ± 0.82 cd
WPL-BatDur2	0.36 ± 0.34 d	21 ± 3.7 cd	0.04 ± 0.11 d	19.61 ± 6.32 ab	15 ± 3.3 ab	4.65 ± 3.12 cd
WPL-MagDur1	0.46 ± 0.76 d	22 ± 5.5 cd	0.28 ± 0.59 d	8.05 ± 2.14 bc	12 ± 0.8 bc	9.11 ± 2.25 bcd
GA-06G[a]	9.94 ± 2.12 ab	12 ± 0.8 a	15.63 ± 5.55 a	28.91 ± 9.19 a	11 ± 1.0 bc	19.84 ± 6.69 a
TifNV-High O/L[a]	11.25 ± 5.37 a	13 ± 1.6 a	12.06 ± 3.65 ab	26.99 ± 14.35 a	11 ± 1.1 bc	15.07 ± 5.60 ab
Bailey[b]	5.98 ± 2.22 bc	14 ± 0.8 ab	7.64 ± 4.78 bc	15.06 ± 7.81 abc	13 ± 2.1 abc	12.98 ± 6.98 abc

Abbreviations: DLA, % diseased leaf area; IP, incubation period; AUDPC, area under the disease progression curve (sporulated lesions/area).
[a]Cultivated peanut—susceptible controls.
[b]Cultivar with wild segments from *A. cardenasii*, partially resistant to ELS.

of 39.5% to 55.0%), and cultivated checks had an average of 45.9% (range of 40.1% to 52.8%). As expected, highly significant differences were observed between the high oleic cultivars, 'TifNV-HO' (PI 680611) and 'TifJumbo' (Holbrook et al., 2024, PI 701814), and low-oleic cultivars, 'Bailey' (Isleib et al., 2011), GA-06G, and Tifrunner ($F = 141.34$, $p < 0.001$). Although not as large, significant differences were also observed between the low-oleic cultivars and the allotetraploids. Interestingly, all induced allotetraploids had much higher protein content than all cultivars tested ($F = 6.54$, $p < 0.001$) (mean protein content of allotetraploid = 32.3%, ranging from 19.0% to 40.1%; mean protein content of cultivars = 20.9%, ranging from 11.1% to 26.6%) (Figure 6). WPL-MagDur1 had the lowest oil content and oleic acid and had the highest protein content (Table 4).

TABLE 3 Results of in vitro evaluation of stem rot (caused by *Agroathelia rolfsii*) of six induced allotetraploids. Peanut cultivars GA-09B and GA-12Y were used as susceptible and resistant controls, respectively.

Genotype/trait	5 DAI	7 DAI	9 DAI	AUDPC
WPL-IpaDur1	1.11 ± 1.24 b	1.64 ± 1.51 ab	3.85 ± 7.03ab	10.02 ± 11.51 ab
WPL-IpaCor1	1.09 ± 0.81 b	1.54 ± 1.42 ab	2.90 ± 3.52 ab	9.06 ± 7.53 ab
WPL-IpaVillo1	0.98 ± 0.88 b	1.53 ± 1.31 ab	2.92 ± 3.29 ab	8.63 ± 7.68 ab
WPL-BatDur1	1.20 ± 0.85 ab	1.72 ± 1.18 ab	3.52 ± 3.56 ab	10.32 ± 6.87 ab
WPL-BatDur2	1.15 ± 0.90 ab	1.89 ± 1.52 ab	3.32 ± 3.74 ab	10.32 ± 8.28 ab
WPL-MagDur1	1.51 ± 1.01 ab	2.13 ± 1.76 ab	4.63 ± 8.01 ab	13.10 ± 12.48 ab
GA-12Y	1.31 ± 1.07 ab	1.51 ± 1.28 ab	1.99 ± 1.73 ab	8.53 ± 6.96 ab
GA-09B	2.27 ± 1.22 a	2.85 ± 1.88 a	3.84 ± 2.52 a	15.40 ± 8.59 a

Note: Means within columns for individual evaluations that are followed by a common letter are not significantly different.
Abbreviations: DAI, days after inoculation; AUDPC, area under the disease progress curve.

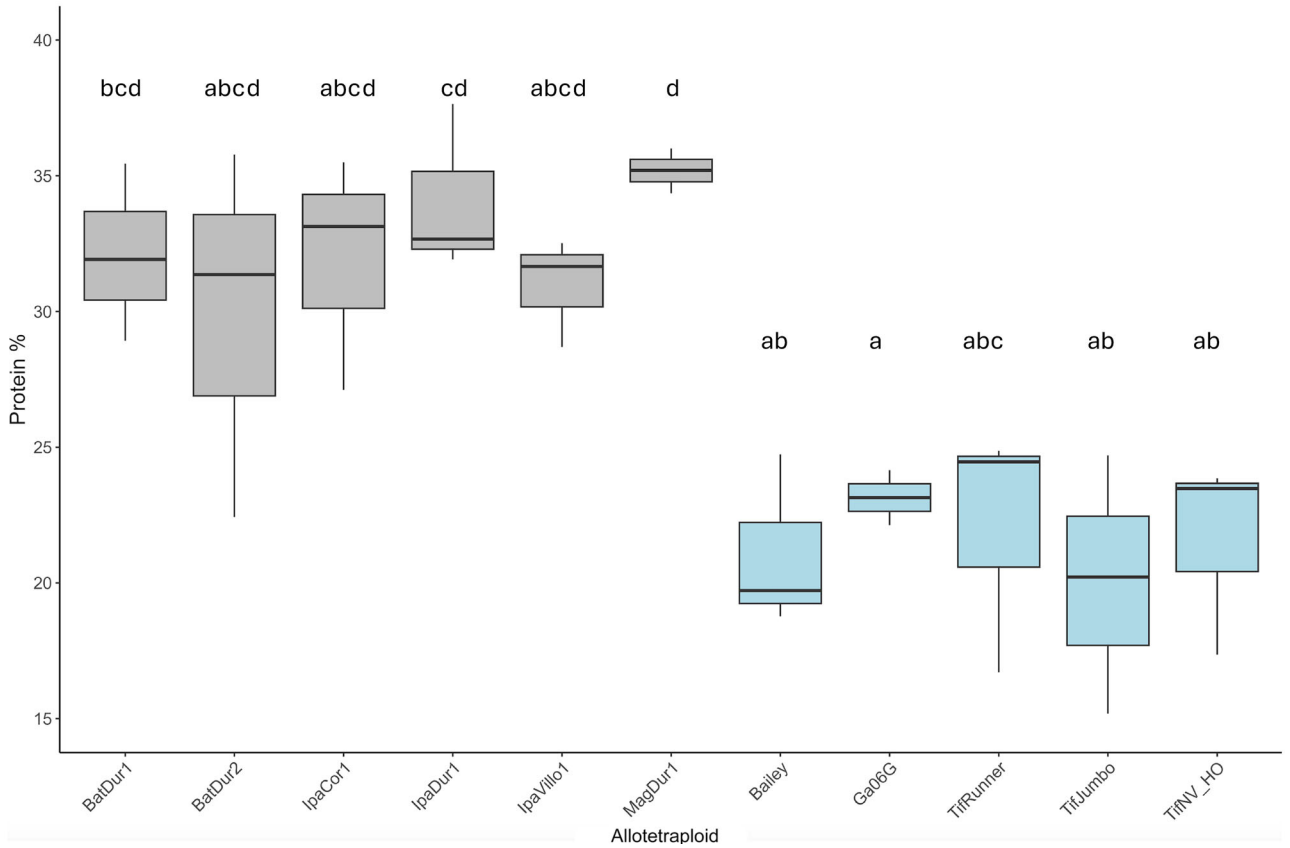

FIGURE 6 Box plot diagrams for seed protein content (as % of total mass). In gray are the induced allotetraploids and in blue, peanut cultivars. Boxes contain 50% of the data points. Bars across boxes represent the median. The top and bottom ends of the whiskers represent the highest and lowest values observed. Values with common letters are not significantly different ($P = 0.05$).

3.5 | Genotyping markers

Polymorphic markers were identified in all chromosomes as specific for WPL-IpaCor1 (247), WPL-IpaDur1 (129), WPL-IpaVillo1 (263), WPL-BatDur1 (212), WPL-BatDur2 (297), and WPL-MagDur1 (318) (Supplemental File 1). The markers that distinguish these allotetraploids from the panel of peanut cultivars are presented in Supplemental File 1. Markers are ordered by chromosome and physical position. This information can be used to design specific primers to identify hybrids and perform genetic mapping.

TABLE 4 Results of quality analyses of seeds of six induced allotetraploids. Oil and protein are expressed as % of total seed mass. Oleic acid is expressed as % total fatty acids.

Genotype/trait	Total oil	Oleic acid	Total protein
	%	%	%
WPL-IpaDur1	44.5 ± 4.7 a	37.4 ± 0.9 de	34.1 ± 3.1 cd
WPL-IpaCor1	43.4 ± 4.3 a	37.1 ± 1.2 de	31.9 ± 4.3 abcd
WPL-IpaVillo1	46.6 ± 2.8 a	38.2 ± 1.2 de	30.9 ± 2.0 abcd
WPL-BatDur1	48.6 ± 3.0 a	34.3 ± 4.8 e	32.1 ± 3.3 abc
WPL-BatDur2	47.9 ± 7.9 a	36.9 ± 1.3 de	29.8 ± 6.8 abcd
WPL-MagDur1	41.6 ± 1.1 a	32.1 ± 3.1 e	35.2 ± 0.8 d
Bailey	47.4 ± 4.2 a	49.4 ± 1.5 b	21.1 ± 3.2 ab
GA-06G	49.3 ± 2.8 a	45.8 ± 2.6 bc	19.8 ± 5.9 a
TifNV-HO	42.3 ± 1.2 a	72.8 ± 1.5 a	21.6 ± 3.6 ab
Tifrunner	45.2 ± 2.6 a	42.1 ± 1.1 bc	22.0 ± 4.6 abc
TifJumbo	45.4 ± 4.2 a	77.4 ± 1.9 a	20.0 ± 4.8 ab

Note: Means within columns for individual evaluations that are followed by a common letter are not significantly different.

4 | CONCLUSIONS

Peanut has low genetic diversity and introducing genetic variation from wild species into cultivars requires considerable time, resources, and expertise. Crossing peanut with the wild diploid species results in sterile offspring due to the distinct ploidy levels. Therefore, a key step to the introgression process is making wild alleles available in a tetraploid form, so it is freely crossable with peanut, and this was the goal of this work.

The accession of *A. ipaënsis* used here (K 30076) as female parent for three crosses was very likely a descendant from the same population that donated the B subgenome to *A. hypogaea* (Bertioli et al., 2016). This is indicated by the extraordinary DNA identity between *A. ipaënsis* K 30076 and the B subgenome of peanut (modal values of 99.98%) (Bertioli et al., 2016; Yin et al., 2020). Although *A. ipaënsis* has not been found to possess strong disease resistances, this closeness to the B-subgenome of peanut makes it an ideal bridge for introgressing alleles from A-genome species. The first allotetraploid produced using *A. ipaënsis* was obtained by Fávero et al. (2006) in a seminal paper that describes what can essentially be considered the re-synthesis of peanut. This work was a turning point into the discussion of the progenitor species of peanut and made them the first *Arachis* species to have their full genome sequenced (Bertioli et al., 2016). Because their diploid genomes and the subgenomes of *A. hypogaea* are so close, when crossed with *A. hypogaea*, populations have vigorous, fertile, and phenotypically normal progeny and genetic maps have lower segregation distortion than for some populations of intraspecific crosses (Fonceka et al., 2009; Gautami et al., 2012; Shirasawa et al., 2013). The cross between *A. ipaënsis* and *A. duranensis* was subsequently repeated in the Wild Peanut Lab, using seeds deposited at the USDA National Plant Germplasm System (NPGS). The resulting allotetraploid, WPL-IpaDur1, created with the original progenitors of peanut, is the one with the lowest level of disease resistance (Tables 2 and 3).

Peanut is probably the only known allotetraploid for which the original progenitors still exist and are available in a seed bank. Furthermore, it has a wild counterpart, *A. monticola*, derived from the same original cross (Leal-Bertioli et al., 2021). Now, the resynthesized peanut, WPL-IpaDur1, is also available and in public domain. The set of genotypes *A. ipaënsis*, *A. duranensis*, *A monticola*, IpaDur1 (the proto-peanut), and cultivated peanut, now all available at the NPGR, is a very powerful package for experimentation on the effects of tetraploidization and domestication of any trait of interest. For instance, the previously synthesized allotetraploid IpaDur (Fávero et al., 2006) has served as a model for a basic study on changes of drought tolerance-related physiological and anatomical traits upon hybridization and polyploidization (Leal-Bertioli et al., 2012). It also served as a model for studies on genomic structure and genetic instability and shed light on the early stages of the origin and domestication of peanut (Leal-Bertioli et al., 2021; Nascimento et al., 2018). It is readily crossable with peanut, seed size can be recovered with only one backcross, and progeny show strong transgressive segregation (e.g., Fonceka et al., 2012; Leal-Bertioli et al., 2018). IpaDur can also restore alleles that were lost in peanut polyploidy through tetrasomic recombination (Leal-Bertioli et al., 2018). IpaDur has also been used in breeding with relative ease; it generated six cultivars in Senegal with larger pods and better yield stability than its recurrent parent (Faye et al., 2016). In Brazil, the cultivar 'BRS 425', also derived from IpaDur, has larger seed size and slightly stronger disease resistance than its recurrent parent, and it is highly productive in some peanut growing regions in the south of the country (Pereira et al., 2023; Suassuna et al., 2019; Suassuna et al., 2015). The other five allotetraploids have not been studied to the same degree, but hopefully, their superior resistance in a form that is readily crossable with peanut will make them useful assets in peanut improvement.

Peanut is an important source of protein in peoples' diet, especially in regions where meat protein is scarce. Average protein content in peanut seeds is 26% (Wang et al., 2016). In this study, using some of the main cultivars planted in the United States, the average was 20.9%. Allotetraploids had a range of 29.8% to 35.2%, significantly higher than all cultivars tested. These results indicate the potential of using these synthetics to improve the nutritional value of peanut.

The allotetraploids described here show superior resistance to the diseases tested. We envisage that they will also carry alleles that will provide resistance to other pests and diseases not evaluated in this work. They all have higher protein

content, which makes them valuable for the food market by offering nutritionally enhanced options that cater to health-conscious consumers seeking protein-rich diets. The creation and seed multiplication of allotetraploids is not a trivial task; painstaking crosses, colchicine treatment with a low recovery rate of tetraploids, and the low seed set are familiar challenges. The availability of these allotetraploids in the NPGS makes it possible for research groups to skip this phase. The markers published will allow hybrids between allotetraploids and peanuts to be easily identified. Our end goal is to create a core collection at the NPGS of wild-derived *Arachis* allotetraploids for the benefit of the peanut research and breeding community worldwide. The production and distribution of these allotetraploids is a step towards this goal.

5 | AVAILABILITY

Seeds of WPL-BatDur1, WPL-BatDur2, WPL-MagDur1, WPL-IpaCor1, WPL-IpaDur1, and WPL-IpaVillo1 have been deposited in the USDA-ARS National Plant Germplasm System (Fort Collins), and in the USDA Plant Genetic Resources and Conservation Unit (Griffin), where it is available for research purposes immediately upon publication. There is limit on the seeds to be distributed. The authors request that appropriate recognition be made if these materials contribute to the development of new germplasm, breeding line, cultivar and/or scientific publication.

AUTHOR CONTRIBUTIONS

Soraya Bertioli: Conceptualization; data curation; formal analysis; funding acquisition; investigation; project administration; supervision; writing—original draft; writing—review and editing. **Mark Hopkins**: Investigation; methodology; writing—review and editing. **Jennifer Leverett**: Investigation; writing—review and editing. **Maricel Gonzales**: Investigation; methodology; writing—review and editing. **Yun-Ching Tsai**: Investigation; methodology; writing—review and editing. **Daniel Matusinec**: Investigation; methodology; writing—review and editing. **Brandon Tonnis**: Formal analysis; investigation; writing—review and editing. **Ana Claudia Araujo**; Investigation; methodology; visualization; writing—review and editing. **David Bertioli**: Conceptualization; funding acquisition; project administration; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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