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Origin of triploid *Arachis pintoi* (Leguminosae) by autopolyploidy evidenced by FISH and meiotic behaviour

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• *Background and Aims* Polyploidy is a dominant feature of flowering-plant genomes, including those of many important crop species. *Arachis* is a largely diploid genus with just four polyploid species. Two of them are economically important: the cultivated peanut and *A. glabrata*, a tropical forage crop. Even though it is usually accepted that polyploids within papilionoid legumes have arisen via hybridization and further chromosome doubling, it has been recently suggested that peanut arose through bilateral sexual polyploidization. In this paper, the polyploid nature of the recent, spontaneously originated triploid cytotype of the tropical lucerne, *A. pintoi*, was analysed, and thereby the mechanism by which polyploids may arise in the genus.
• *Methods* Chromosome morphology of 2*x* and 3*x A. pintoi* was determined by the Feulgeńs technique and the rDNA sites were mapped by FISH. To investigate whether polyploidization occurred by means of unreduced gametes, a detailed analysis of the microsporogenesis and pollen grains was made.
• *Key Results* The 2*x* and 3*x* plants presented 9m + 1sm and a satellited chromosome type 2 in each haploid genome. Physical mapping revealed a cluster of 18S–26S rDNA, proximally located on chromosome 6, and two 5S rDNA loci on chromosomes 3 and 5. Diploid plants presented 10II in meiosis while trivalents were observed in all triploids, with a maximum of 10III by cell. Diploid *A. pintoi* produced normal tetrads, but also triads, dyads and monads. Two types of pollen grains were detected: (1) normal-sized with a prolate shape and (2) large ones with a tetrahedral morphology.
• *Conclusions* Karyotype and meiotic analysis demonstrate that the 3*x* clone of *A. pintoi* arose by autopolyploidy. The occurrence of unreduced gametes strongly supports unilateral sexual polyploidization as the most probable mechanism that could have led to the origin of the triploid cytotype. This mechanism of polyploidization would probably be one of the most important mechanisms involved in the origin of economically important species of *Arachis*, either by triploid bridge or bilateral sexual polyploidization.

Key words: *Arachis pintoi*, autotriploid, Caulorrhizae, chromosome pairing, fluorescent *in situ* hybridization, unreduced gametes.

INTRODUCTION

The genus *Arachis* (Leguminosae) is native to South America and has 80 formally recognized species (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). These entities have been assembled into nine sections according to morphology, geographic distribution and cross compatibility. Among them, the Caulorrhizae section includes two species: *A. pintoi* and *A. repens*. These species are diploids with 2*n* = 2*x* = 20 (Fernández and Krapovickas, 1994) and grow naturally in the states of Goiás, Bahia and Minas Gerais in Brazil. *Arachis pintoi*, besides having good seed set in most accessions, is adapted for clonal propagation by means of stolons. *Arachis repens* rarely sets seeds but is easily propagated from stolons.

Arachis pintoi produces high quality forage (Grof, 1985), and several cultivars have been released in Colombia and Brazil. Among them 'Maní Forrajero Perenne' (Rincón et al., 1992) is the most widely cultivated in the tropics and is usually referred to as the tropical lucerne. Many cultivars

and wild accession of this species are highly adapted to acid and low fertility soils, have a high nutritive value, and persist among aggressive grasses like *Brachiaria* and *Panicum* (Lascano and Thomas, 1988)

A clone with higher forage productivity was isolated from a cultivated plot of diploid *A. pintoi* 'Maní Forrajero Perenne' in Costa Rica. Chromosome counts on material from this clone have shown that it is triploid (Peñaloza et al., 1996), but the clone has not been analysed further. In spite of this paucity of data, the triploid cytotype is largely accepted as having arisen by autopolyploidy, but there is no evidence in this regard. Therefore, the first objective of this study was to determine the nature of this triploid accession of *A. pintoi*.

One of the ways to determine the genome constitution of polyploids is by analysing karyotypes using classical techniques, and for those groups with small, similar chromosomes, homologies have been established by physical mapping of rDNA genes (Jiang and Gill, 1994). Particularly, in *Arachis* species, the ability to detect ribosomal genes by FISH has increased the number of chromosomal markers, allowing the

establishment of homologies for more chromosomes than was possible with classical cytogenetics (Robledo and Seijo, 2008; Robledo *et al.*, 2009).

Another powerful tool to investigate whether a species is an auto- or allopolyploid is by analysing meiotic behaviour and determining meiotic chromosomal associations during diakinesis or metaphase I (Jauhar and Joppa, 1996). According to the number and type of chromosome associations (univalents, bivalents, trivalents, tetravalents), it is possible to determine the polyploid status of the species under study (Stebbins, 1947).

Therefore, to attain the first objective, the following were analysed: (*a*) the chromosome homologies between the $2x$ and $3x$ cytotypes of *A. pintoi* by determining the karyotype formulae using Feulgen's technique and by mapping the 5S and the 18S–25S rDNA sites using FISH; and (*b*) the degree of homology of the triploid chromosome complements by establishing the meiotic configurations during meiotic metaphase I.

In the genus *Arachis*, there are four polyploid species. Two are economically important: cultivated peanut, *Arachis hypogaea*, an allotetraploid that originated around 5000 years BP (Bonavia, 1982); and *A. glabrata*, a forage crop in the southern United States and Australia. Even though *A. glabrata* has been considered to be an autopolypoid with different degrees of diploidization, its polyploid nature is still unknown. *Arachis hypogaea*, is usually accepted to have arisen via hybridization and further chromosome doubling. However, recently we proposed that this cultigen arose through bilateral sexual polyploidization as the mechanism of origin for this cultigen (Seijo *et al.*, 2007). The recent, spontaneous origin of the triploid *A. pintoi* provides a unique opportunity to study the mechanism by which polyploids may arise in the genus and the genomic processes that occurred after that event.

The analysis of meiotic behaviour, sporads and pollen grains in other plant groups has revealed the occurrence of $2n$ pollen and the possibility that new cytotypes arise by sexual polyploidization (Bretagnolle, 2001; Jauhar, 2007). In the genus *Arachis*, the formation of unreduced gametes has been reported in a triploid artificial hybrid, *A. hypogaea* × (*A. cardenasii* × *A. diogoi*) (Simpson and Davis, 1983); however, there are no records of such an occurrence in diploid taxa.

In this context, the second objective was to obtain evidence to understand the mechanism involved in the origin of the triploid *A. pintoi* to infer the mechanism of polyploid formation in *Arachis* as a whole. For this purpose, the microsporogenesis and pollen grains (morphology and size) of the diploid accession were analysed in the search for $2n$ gametes.

MATERIALS AND METHODS

Plant material

The material studied was the cultivar *Arachis pintoi* Krapov. & W.C. Gregory 'Maní Forrajero Perenne': CIAT 17434 with diploid cytotype ($2n = 2x = 20$), derived from the original botanical collection, Gregory and Krapovickas 12787; and triploid plants ($2n = 3x = 30$), originally from a plot of diploid 'Maní Forrajero Perenne' from Costa Rica. Both materials

are in the germplasm bank of the Instituto de Botánica del Nordeste, Corrientes, Argentina.

Chromosome preparations and Feulgen staining in 2x and 3x A. pintoi

Plants of the two cytotypes were obtained from stolons and grown in pots. Roots 10–15 mm long were directly collected from those plants, pretreated with 2 mM L^{-1} 8-hydroxyquinoline for 3 h at room temperature (Fernández and Krapovickas, 1994) and then fixed in 3 : 1 absolute ethanol : glacial acetic acid for a minimum of 12 h at 4 °C. Chromosomes were stained using Feulgen's technique, then squashed in a drop of 2 % acetic orcein.

Fluorescent in situ hybridization in 2x and 3x A. pintoi

Chromosome spreads. Until fixation, the steps were the same as those just described, then somatic chromosome spreads were prepared according to Schwarzacher *et al.* (1980). Root apices were digested in 1 % (w/v) cellulose (from *Trichoderma viridae*, Onozuka R-10; Serva, Heidelberg, Germany) plus 10 % (v/v) pectinase dissolved in 40 % glycerol (from *Aspergillus niger*; Sigma, St Louis, Missouri, USA) in 0·01 M L^{-1} citrate buffer, pH 4·8, at 37 °C for 2 h. Subsequently, the meristematic cells were removed from the root tips and squashed in 45 % acetic acid. After removal of the coverslip with carbon dioxide, the slides were air-dried, aged for 1–2 d at room temperature, and then kept at −20 °C until use.

Probe labelling and fluorescent in situ hybridization. The 5S rDNA and 18S–26S rDNA loci were localized using probes pA5S, pA18S and pA26S isolated from genomic DNA of *A. hypogaea* (Robledo and Seijo, 2008) and labelled by nick translation with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) or biotin-11-dUTP (Sigma). Pretreatment of slides, chromosome and probe denaturation, conditions for the *in situ* hybridization (hybridization mixes contained DNA probes at a concentration of 2·5–3·5 ng μL^{-1}, with a stringency to allow sequences with 80–85 % identity to remain hybridized), post-hybridization washing, blocking and indirect detection with fluorochrome-conjugated antibodies were performed according to Moscone *et al.* (1996). The first set of antibodies consisted of mouse anti-biotin (Dakopatts, Dako, Carpinteria, CA, USA) and sheep anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC) (Boehringer Mannheim). The second set of antibodies consisted of rabbit anti-mouse conjugated to tetramethyl-rhodamine isothiocyanate (TRITC) (Dakopatts) and FITC-conjugated rabbit antisheep (Dakopatts). Preparations were counterstained and mounted with Vectashield medium (Vector Laboratories, Burlingame, CA, USA) containing 2 mg mL^{-1} of 4′,6-diamidino-2-phenylindole (DAPI).

Fluorescence microscopy and image acquisition. Chromosomes were viewed and photographed with a Leica DMRX fluorescence microscope (Leica, Heerbrugg, Switzerland) equipped with a computer-assisted Leica DC 350 digital camera system. Red, green, and blue images were captured in black and white using appropriate filters for TRITC, FITC and DAPI

excitation, respectively. Digital images were processed with Photoshop, version 7·0 (Adobe, San Jose, CA, USA).

Meiotic analysis

Inflorescences of 2*x* and 3*x* *A. pintoi* were fixed in 3 : 1 absolute ethanol:acid acetic for 24 h, transferred to 70 % ethanol and refrigerated until use. Pollen mother cells (PMCs) were then stained in a drop of 2 % lactopropionic orcein. Representative permanent slides were made using Euparal as mounting medium.

Production of 2n gametes in 2x A. pintoi

Sporads were screened, and the occurrence of 2*n* gametes in dyads and triads was recorded. Unreduced pollen grains were identified by a detailed examination of pollen throughout the range of sizes produced by each individual (Bretagnolle and Thompson, 1995). This method is based on the fact that cell (pollen) size increases as DNA content increases (Bennett, 1972). Pollen samples were obtained from two flowers (each containing a mixture of pollen grains from different anthers) in each of the five individual plants analysed and stained by 1 : 2 % acetocarmine : glycerin. These slides were used for estimating the pollen viability and to determine the mean pollen size.

Analysis of pollen-grain morphology by scanning electron microscopy (SEM)

Pollen samples were obtained from fresh flowers of plants grown under greenhouse conditions. To analyse pollen shape, grains were acetolysed according to the procedure suggested by Erdtman (1966). The acetolysed grains were first washed in 96 % alcohol, and then in absolute alcohol. The samples were air-dried in Petri dishes, sputtered with gold–palladium and finally observed with a JEOL 5800 LV scanning electron microscope at 15 kV. The terminology used to describe pollen follows Punt *et al.* (2007).

RESULTS

Karyotypic features of 2x and 3x A. pintoi

Classical cytological analysis demonstrated that the diploid clone has 18 metacentrics (m) and two submetacentrics (sm), while the triploid presents 30 chromosomes (Fig. 1) arranged in 27m + 3sm. Two satellited chromosomes (SAT chromosomes) type 2 were observed in the diploid and three in the triploid.

Physical mapping of rDNA genes using FISH revealed clusters of 18S–26S rDNA exclusively localized in the secondary constriction of the SAT chromosomes; these signals were located near the centromere on the short arms of metacentric pair 6 in both the 2*x* and the 3*x* plants (Fig. 2). In all metaphases, 18S–26S rDNA loci presented fluorescent signals of similar intensity. *In situ* hybridization with the 5S rDNA probe revealed two signals of similar intensity per haploid complement. These loci were localized on the short arms of
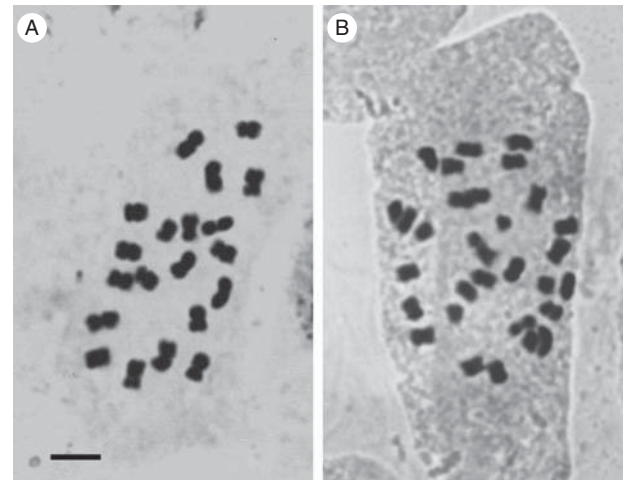

FIG. 1. Somatic chromosomes of *A. pintoi* with Feulgen's staining: (A) diploid cytotype with 2*n* = 20 chromosomes; (B) triploid cytotype with 2*n* = 30 chromosomes. Scale bar = 5 μm.
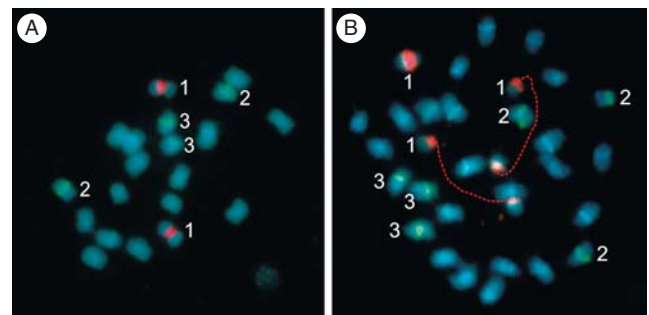

FIG. 2. Somatic chromosomes of (A) 2*x* and (B) 3*x* plants of *A. pintoi* after double fluorescent *in situ* hybridization (FISH) showing yellow-green fluorescein isothiocyanate (FITC) signals from 5S rDNA probe (two chromosomes per haploid complement, indicated by numbers 2 and 3) and red tetramethyl-rhodamine isothiocyanate (TRITC) signals from the 45S rDNA probe (indicated by number 1). Chromosomes were counterstained with DAPI (light blue) after the FISH. Homologous chromosomes were indicated with same numbers.

metacentric chromosomes 3 and 5, both in diploid (Fig. 2A) and triploid plants (Fig. 2B).

The 2*x* clones always had two chromosomes with active 18S–26S rDNA clusters (i.e. those that in *Arachis* characteristically remained uncondensed at metaphase), while the number in the 3*x* plants varied between individuals or even between cells of the same plant. Three active clusters were observed in 31 % of the cells, two in 61 % and one in 8 % of them.

Microsporogenesis in triploid plants

Different configurations in the PMCs at diakinesis/metaphase I were found (Table 1); 8III + 2II + 2I was the most frequent (in approx. 40 % of the cells). Trivalents were observed in 100 % of the analysed cells in diakinesis/metaphase I at frequencies that ranged from one to ten per cell (Fig. 3A). The percentage of cells with ten trivalents was 18·75 %

TABLE 1. *Meiotic behaviour of 3*x A. pintoi *in different meiotic phases (metaphase I, anaphase I, telophase I, prophase II, metaphase II and anaphase II) and sporad analysis*

Phase	No. of PMCs	%
Metaphase I		
10III	3	18·75
9III + 1II + 1I	5	31·25
8III + 2II + 2I	7	43·75
Out-of-plate chromosomes	1	6·25
Anaphase I		
Normal (regular segregation of chromosomes)	3	33·33
With one laggard chromosome	3	33·33
With two laggard chromosomes	2	22·22
With five laggard chromosomes	1	11·11
Telophase I		
Normal (all chromosomes in two nuclei)	15	99·99
Out-of-nucleus chromosomes	1	0·01
Prophase II (number of chromosomes per nucleus)		
15/15	17	58·62
14/16	8	27·58
13/17	1	3·45
18/12	1	3·45
19/11	2	6·90
Metaphase II		
Normal (all chromosomes in two plates)	7	77·77
Out-of- plate chromosome (1)	1	11·11
Out-of- plate chromosomes (2)	1	11·11
Anaphase II		
Normal (all chromosomes segregate in two spindles)	18	81·82
With four laggard chromosomes	1	4·54
With two laggard chromosomes	1	4·54
With six poles	2	9·10
Sporads		
Tetrads	362	97·05
Tetrads with two larger size microspores	1	0·27
Tetrads with micromicrospore	8	2·14
Triads	1	0·27
Dyads	1	0·27

(Table 1), and the mean frequency of trivalents per cell was 8·67 (Table 2).

Meiotic irregularities such as out-of-plate and out-of-nuclei chromosomes, laggard chromosomes, and cytomixis (i.e. migration of chromatin between meiocytes through cytoplasmic channels) were observed in different phases of meiosis I and II (Fig. 3B–D) and are summarized in Table 1. Segregation at anaphase I and II was irregular, leading to the formation of unbalanced gametes (Fig. 3D–E). Variation in pollen size and a high percentage of unstained pollen (57·53 %) was recorded in the triploid clone after carmine–glycerine staining (Fig. 3F).

Microsporogenesis of the diploid plants

Meiotic analysis of the diploid cytotype showed the presence of 10II per cell (Fig. 4A) in all the PMCs at metaphase I. Although bivalent formation was regular, some irregular phenomena were observed in cells of different phases (Table 3). Out-of-plate bivalents were found in 14·49 % of the metaphase I cells (Fig. 4B), and chromosome bridges were observed in 0·99 % of cells in ana-telophase I

(Fig. 4D). Cytomictic processes were observed in 5·80 % of the metaphases and in 2·97 % of the telophases I (Fig. 4E). The sporads analysis showed that, in addition to the expected normal tetrads, triads, dyads and monads were formed as a result of the meiotic division (Fig. 4F).

Pollen grain analysis by SEM revealed that pollen grains were a mixture of 91·34 % prolate tricolpate reticulate grains (Fig. 5A), 6·87 % of tetrahedral grains (Fig. 5B) and 1·79 % of unviable grains.

Average pollen grain viability was 93·36 %. Light microscopy analysis of pollen size showed a bimodal distribution (Fig. 6). Based on this result, pollen grains were considered normal when their size ranged between 27·2 and 38·8 μm, and large when the size was above 39·44 μm. With this criterion, the mean size of normal pollen was 32·13 μm, and the frequency was 93·13 %. Large pollen grains with a mean size of 45·7 μm were observed at a frequency of 6·87 %.

DISCUSSION

In the present study, the nature of a spontaneous triploid of *A. pintoi* was investigated by analysing somatic chromosomes and meiotic behaviour in both 2*x* and 3*x* plants. The analysis of the sporads and pollen grains of diploid plants provided data to reconsider mechanism involved in the origin of polyploid taxa in *Arachis*.

Evidence of autopolyploidy

The karyotype formula 18m + 2sm found for the diploid *A. pintoi* with SAT chromosome type 2 agrees with results previously published for the species (Fernández and Krapovickas, 1994). The fact that the karyotype formulae and the SAT chromosomes per haploid complement found in the triploid plants were exactly the same as those observed for the 2*x* clone is evidence that the 3*x* has three chromosome sets identical to those observed in the 2*x*. This observation was confirmed by the physical localization of rDNA by FISH, which showed the same number, size and distribution of the 5S and 18S–26S rDNA loci in each haploid complement of the diploid and triploid plants.

The high frequency of trivalents in all the cells analysed and the presence of up to ten trivalents in diakinesis/metaphase I of the triploid plants indicate a high degree of homology between their three complements. Therefore, the bulk of our evidence demonstrates that the triploid clone of *A. pintoi* originated by autopolyploidy. Considering the genome nomenclature proposed by Smartt and Stalker (1982) for *Arachis* species, the genome constitution of this clone would be CCC.

Origin of triploid plants

Polyploidization may be either asexual by somatic chromosome doubling, or sexual through the formation of *2n* gametes. As opposed to the early belief of the spontaneous doubling of somatic chromosomes as the main mechanism of polyploid origin, Harlan and De Wet (1975) suggested that polyploids in the angiosperms originated primarily from the union of *2n* gametes. More recently, unreduced gametes were considered
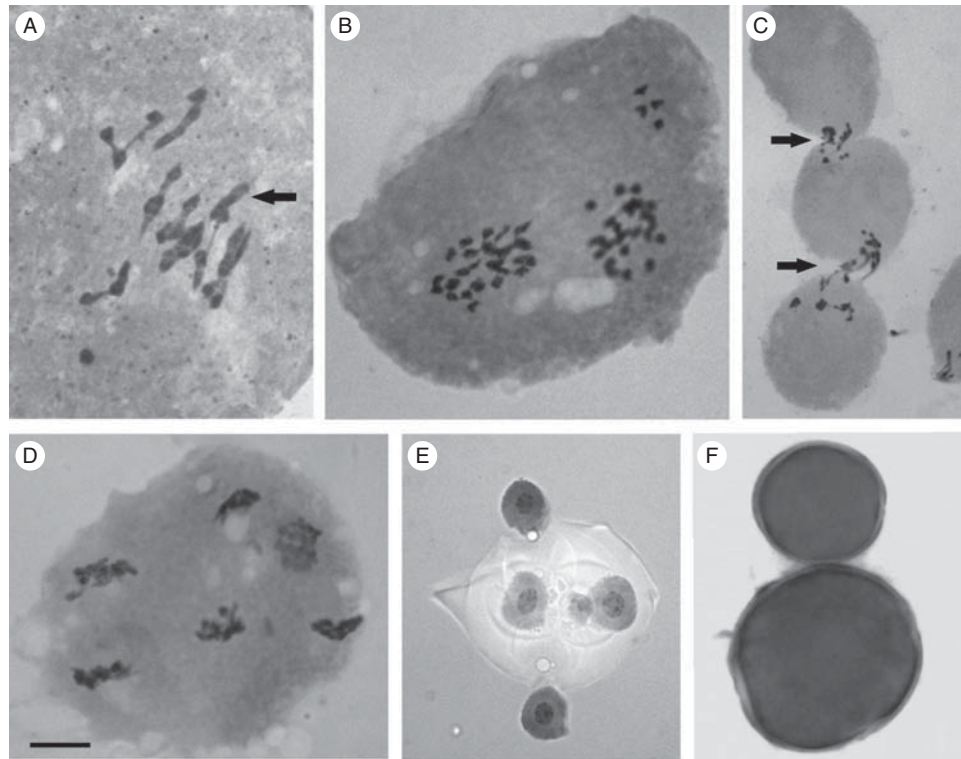
FIG. 3. Microsporogenesis and pollen grains in $3x$ plants of *A. pintoi*: (A) meiotic behaviour in metaphase I, 9III + 1II + 1I (arrow indicates the bivalent); (B) four chromosomes out of the prophase II nuclei; (C) cytomixis (arrows indicate chromatin migration between PMCs through cytoplasmatic channels); (D) six poles in telophase II; (E) pentad with a micromicrospore; (F) two different sizes of pollen grains (large and normal pollen). The scale bar in (D) applies to all the images, as follows: (A, B, D) = 5 μm; (C, E) = 7·5 μm; (F) = 15 μm.

TABLE 2. *Meiotic chromosome associations, mean, range of variation and standard error (s.e.) in triploid cytotype of* A. pintoi

Statistic	Univalents	Bilavents	Trivalents
Mean	1·33	1·33	8·67
Range	0−2	0−2	8−10
s.e.	0·22	0·22	0·23

a dominant process involved in the origin of polyploid plants (Veilleux, 1985; Bretagnolle and Thompson, 1995).

The presence of monads, dyads and triads and large pollen grains constitutes direct evidence that *A. pintoi* produced $2n$ gametes. Whereas monads may potentially yield pollen grains with a quadruplicate chromosome number $4n = 40$, dyads might give rise to two grains of doubled number $2n = 20$, and triads to an unreduced pollen grain $2n = 20$, and two pollen grains with reduced chromosome number $n = 10$. According to Darlington's criterion (Darlington, 1937), a pollen grain is considered unreduced when its size is 1·2–1·3 times larger than n pollen. Therefore, the observations that among the stained pollen the average size of the larger ones was 1·4 times larger than normals also confirmed that unreduced pollen grains were produced by the $2x$ *A. pintoi*.

The morphology of normal-sized pollen found here agrees with that described previously for several *Arachis* species (Pire, 1974; Chaturvedi *et al.*, 1990). Tetrahedral morphology, observed for the large grains, has been reported for *Lotus tenuis* (Rim and Beuselink, 1996) in which the n pollen grains are globose-prolate, but the $2n$ pollen grains are tetrahedral. Therefore, the morphological analysis of pollen grains may also support production of unreduced gametes by diploid plants of *A. pintoi*.

The presence of unreduced gametes in these plants suggests that they have the potential to produce autotriploid plants by unilateral sexual polyploidization via the union of a normal gamete (n) with an unreduced one ($2n$). Several studies of spontaneous triploids indicate that many of the gametes produced by autotriploids are not functional, because they possess aneuploid, unbalanced chromosome numbers. However, triploids generate small numbers of euploid (x, $2x$) gametes and can also produce $3x$ gametes via nonreduction (Ramsey and Schemske, 1998). Therefore, they may constitute a bridge to produce higher polyploids, like tetraploids and hexaploids by self-fertilization or backcrossing to diploids (Ramanna and Jacobsen, 2003). The finding of unreduced gametes in *A. pintoi* supports the hypothesis that the allotetraploid *A. hypogaea* could have also arisen by sexual polyploidization, as suggested by Seijo *et al.* (2007), and that it may be a general mechanism of polyploid formation in *Arachis*.

The mechanisms that may give rise to unreduced gametes can be attributed to different failures during the meiotic process. Meiotic irregularities associated with the appearance of $2n$ gametes are prevalently due to mutations in the genes
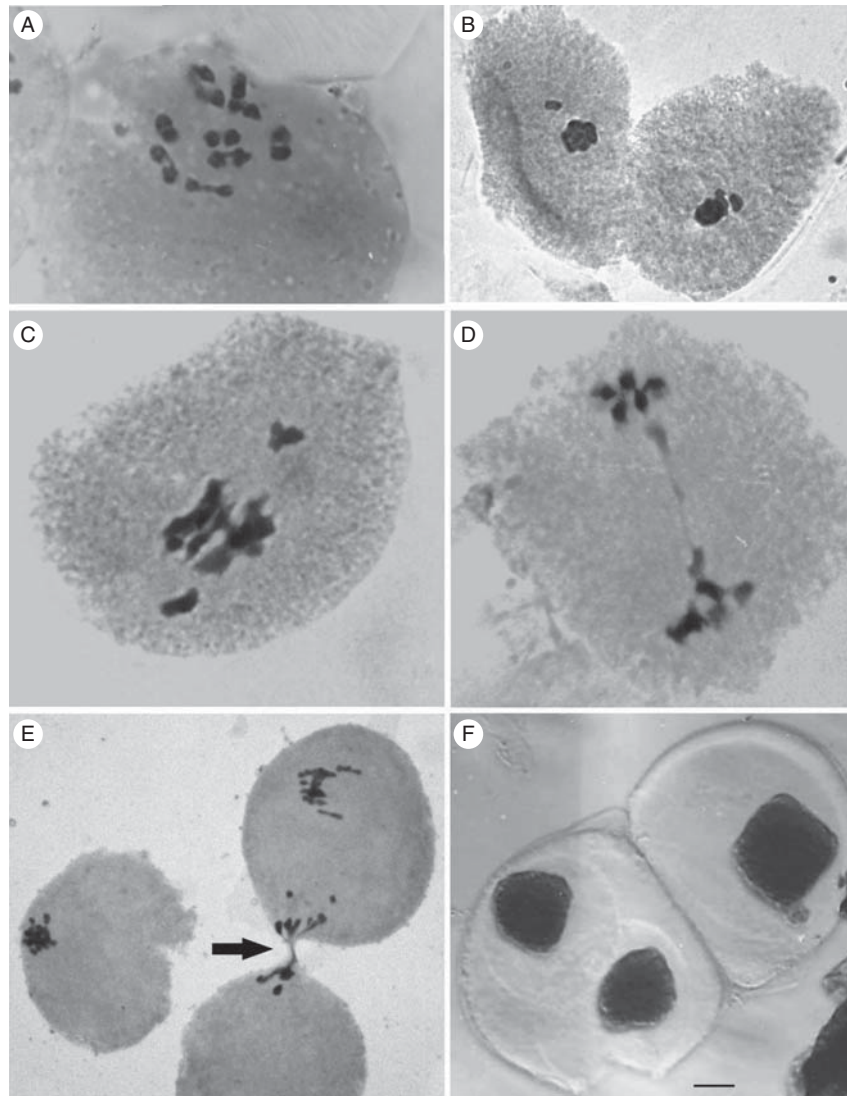
FIG. 4. Microsporogenesis in diploid plants of *A. pintoi*: (A) metaphase I with 10II; (B) out-of-plate chromosomes in metaphase I; (C) metaphase I showing bivalents with early migration to the poles; (D) chromosome bridge in telophase I; (E) cytomixis (arrow indicates chromatin migration between PMCs through cytoplasmatic channels); (F) dyad (left) and monad (right). The scale bar in (F) applies to all the images, as follows: (A, B, D) = 5 μm; (C, E) = 7·5 μm; (F) = 15 μm.

that regulate chromosome pairing, spindle formation and cyto-kinesis (Falistoco *et al.*, 1995). Unreduced gametes may result from a process called nuclear restitution; these have been named first-division restitution and second-division restitution, and result from a failure to divide during the first and the second meiotic divisions, respectively (Bretagnolle and Thompson, 1995). This mechanism is considered a potent force in the evolution of some groups of plants, e.g. the *Triticeae* group (Jauhar, 2007) and *Solanum* (den Nijs and Peloquin, 1977). Although first-division restitution has been reported for an interspecific triploid hybrid of *Arachis* (Simpson and Davis, 1983), nuclear restitution was not evident here during the meiotic division of 2*x A. pintoi*.

Another mechanism that could give rise to unreduced gametes is cytomixis (Falistoco *et al.*, 1995). It has been reported that, as a consequence of this phenomenon, meiocytes that have either fewer or more chromosomes than in normal

TABLE 3. *Meiotic behaviour of* 2x *A.* pintoi *in different phases (metaphase I, telophase I, telophase II) and sporad analysis*

Phase	No. of CMPs	%
Metaphase I		
20II	55	79·71
Out-of-plate bivalent	10	14·49
Cytomixis	4	5·80
Telophase I		
Normal (all chromosomes in two nuclei)	97	96·04
Bridge in anaphase	1	0·99
Cytomixis	3	2·97
Telophase II		
Normal (all chromosomes in four nuclei)	20	100
Sporads		
Tetrad	399	91·94
Triads	2	0·46
Dyads	2	0·46
Monads	31	7·14

cells can be formed. Occasionally, either empty meiocytes or meiocytes with a duplicate number of chromosomes have been reported (Bellucci *et al.*, 2003). It is very probable that the meiocytes without chromatin die, but that those with a double number of chromosomes, or almost double, could lead to the formation of *2n* gametes. This mechanism is considered by some authors to be of evolutionary importance for plants (Zheng *et al.*, 1987; Falistocco *et al.*, 1995). In the present work, the percentages of cells having cytomictic

phenomena (8·77 %), abnormal sporads (8·52 %) and large pollen (6·87 %) were similar in 2x plants, suggesting that cytomixis may be involved in the production of unreduced gametes in *A. pintoi*.

Nucleolar dominance in autopolyploids

Allopolyploidy induces a wider range of changes in gene expression than does autopolyploidy, suggesting that hybridization has greater effects than genome doubling on gene expression and phenotypic variation (Chen, 2007). Nucleolar dominance is a well-known regulatory phenomenon affecting the active number of NOR loci in allopolyploids and diploid hybrids (Pontes *et al.*, 2004). This silencing mechanism depends on an epigenetic switch that, in concert with promoter methylation and histone modifications, results in a self-reinforcing repression cycle of the rRNA genes (Lawrence *et al.*, 2004). In Triticale (a hybrid between wheat and rye), the wheat NORs are active, and the rye NORs are silenced (Pontes *et al.*, 2004). The generality of this epigenetic mechanism is exemplified in synthetic and natural allopolyploids like *Arabidopsis* (Madlung *et al.*, 2005) and *Glycine* (Joly *et al.*, 2004). Evidence of nucleolar dominance in hybrid animals is provided by *Drosophila* (Durica and Krider, 1977), *Xenopus* (Pikaard, 1994) and the mule (Kopp *et al.*, 1986).

However, autopolyploids present additional sets of identical genomes, so that the NOR clusters are in extra doses. Considering that nucleolar dominance occurs in allopolyploid species, one can wonder how many clusters are active in the organization of nucleoli in autopolyploids and whether the extra clusters are inactivated. In this work, 3x *A. pintoi* varied from one to three in the number of active NOR clusters, agreeing with observations for the autopolyploid plant *Silene latifolia* (Siroki *et al.*, 1999) and the frog *Odontophrynus americanus* (Ruiz *et al.*, 1980), in which variable numbers of nucleoli have also been found (Beçak and Kobachi, 2004). Therefore, the number of active NORs can vary in both plant and animal autopolyploid species. Those differences in the numbers of chromosomes involved in nucleolar formation observed in autopolyploids cannot be attributed to the main mechanisms of nucleolar dominance proposed in
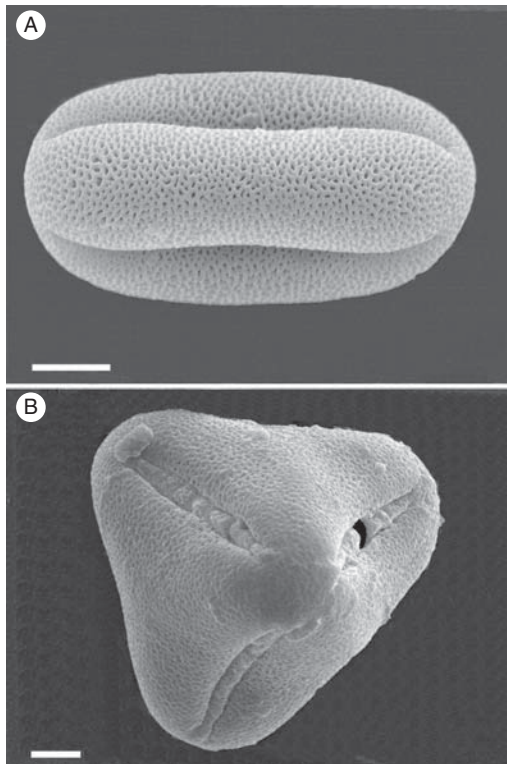
FIG. 5. Pollen grains of 2x *A. pintoi* by SEM: (A) equatorial view of prolate tricolpate reticulate pollen grain; (B) polar view of tetrahedral pollen grain. Scale bars = 10 μm.
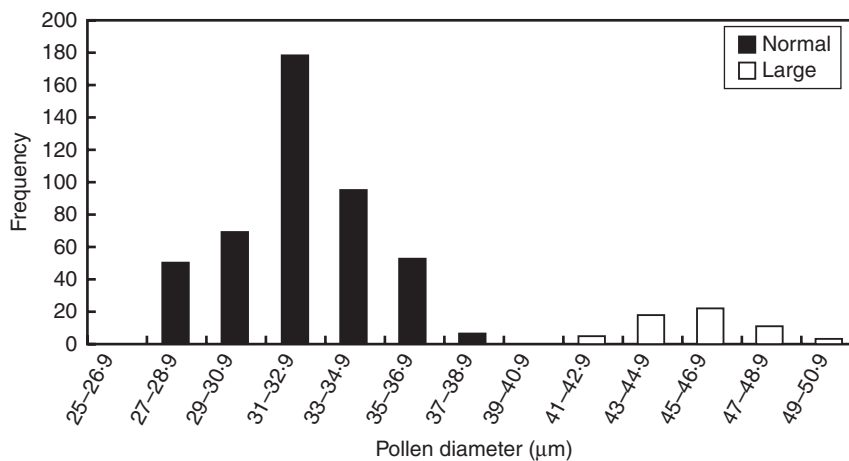
FIG. 6. Distribution of pollen diameter in 2x *A. pintoi*; normal and large pollen as indicated.

allopolyploids, but could probably be the result of a mechanism of gene dosage compensation.

Conclusions

Karyotype analysis by classical staining, physical localization of chromosome markers by FISH and meiotic analysis revealed that the $3x$ cytotype of *A. pintoi* has three identical chromosome complements and demonstrated that this taxa has originated by autopolyploidy. The occurrence of viable unreduced gametes at anthesis of the $2x$ cytotype suggests that they may have a key role in the polyploidization process of this tropical forage legume. These findings may support unilateral or bilateral sexual polyploidization as a main mechanism in the formation of polyploids within the genus. The occurrence of $2n$ pollen may allow *Arachis* breeders to explore new methods for the genetic improvement of peanut and forage species of *Arachis*. Transfer of desired attributes from variable diploid to polyploid species with a narrow genetic base, like peanut, through sexual polyploidization may be more efficient, in time and product delivery, than other analytical breeding schemes proposed for *Arachis* so far.

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