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Segregation for Sexual Seed Production in *Paspalum* as Directed by Male Gametes of Apomictic Triploid Plants

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• *Background and Aims* Gametophytic apomixis is regularly associated with polyploidy. It has been hypothesized that apomixis is not present in diploid plants because of a pleiotropic lethal effect associated with monoploid gametes. Rare apomictic triploid plants for *Paspalum notatum* and *P. simplex*, which usually have sexual diploid and apomictic tetraploid races, were acquired. These triploids normally produce male gametes through meiosis with a range of chromosome numbers from monoploid ($n = 10$) to diploid ($n = 20$). The patterns of apomixis transmission in *Paspalum* were investigated in relation to the ploidy levels of gametes.
• *Methods* Intraspecific crosses were made between sexual diploid, triploid and tetraploid plants as female parents and apomictic triploid plants as male parents. Apomictic progeny were identified by using molecular markers completely linked to apomixis and the analysis of mature embryo sacs. The chromosome number of the male gamete was inferred from chromosome counts of each progeny.
• *Key Results* The chromosome numbers of the progeny indicated that the chromosome input of male gametes depended on the chromosome number of the female gamete. The apomictic trait was not transmitted through monoploid gametes, at least when the progeny was diploid. Diploid or near-diploid gametes transmitted apomixis at very low rates.
• *Conclusions* Since male monoploid gametes usually failed to form polyploid progenies, for example triploids after $4x \times 3x$ crosses, it was not possible to determine whether apomixis could segregate in polyploid progenies by means of monoploid gametes.

Key words: Apomixis, monoploid gametes, *Paspalum notatum*, *Paspalum simplex*, polyploidy, RAPD, SCAR, triploidy.

INTRODUCTION

Apomixis is an asexual mode of reproduction through seeds. Embryos are produced without a female haploid reduction phase and without any contribution of the paternal parent. They may originate from unreduced gametophytes, gametophytic apomixis, or directly from the somatic tissue of nucellus or the integument of the ovule (Nogler, 1984). Gametophytic apomixis involves three main components: (1) failure of meiosis, (2) unreduced embryo sac development and (3) parthenogenetic development of the unreduced egg. In general, the process only occurs in the female part, while the male meiosis generates normal haploid pollen. Many apomictic species are pseudogamous requiring fertilization to initiate endosperm development. It is well known that gametophytic apomixis is related to polyploidy. Almost all apomictic plants that have been cytologically examined were polyploid, and the majority tetraploid (Savidan, 2000). A few apomictic diploid exceptions are mentioned in the literature, although some remain rather doubtful (Holm and Ghatnekar, 1996). Furthermore, most of the reported apomictic diploids were dihaploids, obtained by haploid parthenogenesis from apomictic tetraploids (de Wet, 1968, 1971; Savidan and Pernès, 1982; Kojima and Nagato, 1997; Naumova *et al.*, 1999), and usually described as weak and sterile (Nogler, 1982; Leblanc *et al.*, 1996; Bicknell and Borst, 1997). Unreduced embryo sacs have been observed in ovules from diploids as well as from artificially induced tetraploids indicating that the apomixis factor(s) was present in diploid races (Savidan, 1982; Quarin and Norrmann, 1987; Norrmann *et al.*, 1989; Quarin *et al.*, 2001). However, asexual progeny from those diploids bearing additional aposporous embryo sacs has not been demonstrated.

With a few exceptions, apomixis is poorly represented among crop species. The introduction of apomixis into sexual crops is an important goal in apomixis research (Hanna, 1995). Apomixis has not yet been transferred from a wild species to any sexual relative crop (Savidan, 2000). The diploid status of many crops is an important barrier in achieving this goal. Little is known about the reasons for the absence, or non-expression of this reproductive mode in diploids. However, two hypotheses have been postulated to explain the almost complete absence of apomixis at the diploid level. One assumes that the factor responsible for apomixis could not be transmitted through monoploid gametes; therefore, apomixis could not be recovered in diploids (Nogler, 1982). In *Ranunculus auricomus*, a species with aposporous apomixis, Nogler (1984) has demonstrated that apomixis is monogenic and dominant, and it could only be transmitted in the heterozygous state through diploid or polyploid gametes. The dominant allele responsible for apomixis is lethal in its homozygous

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state; therefore, it could not be transmitted through monoploid gametes. The second hypothesis supposes that a dosage requirement is necessary for apomixis to be expressed (Mogie, 1988; Quarin *et al.*, 2001). According to this hypothesis, the absence of apomixis in wild diploids is due to a lack of expression instead of non-transmission. Mogie (1988) suggested that more than two copies of the apomixis allele are necessary for the expression of the trait. Grimanelli *et al.* (1998) obtained results in maize–*Tripsacum* hybrids that supported Nogler's hypothesis (Nogler, 1982). Apomixis in *Tripsacum* is linked to a segregation distorter-type system that promotes the elimination of the apomixis alleles when transmitted through haploid gametes (Grimanelli *et al.*, 1998). Meanwhile, contrasting evidence has been found in *Paspalum* both supporting and rejecting Mogie's hypothesis. A few tetraploids generated by colchicine treatment of diploid tissue were determined to be facultative apomicts indicating the presence, without expression of the apomixis gene(s) in diploid plants (Quarin and Hanna, 1980; Quarin *et al.*, 2001), thus supporting Mogie's hypothesis. On the other hand, aposporous apomixis in *P. notatum* (Martinez *et al.*, 2001), and *P. simplex* (Cáceres *et al.*, 2001) was determined to be inherited as a single Mendelian factor, present in simplex condition (+- - -) in autotetraploid accessions, rejecting Mogie's hypothesis and partially supporting Nogler's hypothesis. Bicknell *et al.* (2000) observed in *Hieracium* that apomixis was transmitted not only by diploid gametes but also by haploid gametes when the recovered progeny were polyploid. They proposed that the absence of apomixis in diploids was caused by selection against the survival of diploid zygotes, rather than against the haploid gametes as had been noted in other systems (Bicknell *et al.*, 2000).

Paspalum is a grass genus native to the New World with several important forage species. Most species are polyploids, and among them, tetraploids are predominant (Quarin, 1992). Species containing apomictic polyploids as well as sexual diploids are frequently found. Apospory is the most common type of apomixis in the genus. *Paspalum notatum* and *P. simplex* are the two species of the genus where apomixis has been most extensively studied.

Paspalum notatum is a rhizomatous species widely distributed in native grasslands from central Mexico to Argentina (Chase, 1929). Different ploidy levels were reported for this species (Burton, 1946; Gould, 1966; Tischler and Burson, 1995). Sexual diploids are cross-pollinated due to self-incompatibility (Burton, 1955), while the tetraploid races reproduce by obligate apomixis and are pseudogamous (Burton, 1948). It has been reported that apospory in tetraploid *P. notatum* is controlled by a major dominant single locus (Martínez *et al.*, 2001). However, the trait was transmitted in a non-Mendelian fashion due to a distortion in the segregation ratio. In addition, several molecular markers completely linked to the apomixis locus were detected (Martínez *et al.*, 2003; Stein *et al.*, 2004).

Paspalum simplex is a warm-season grass found in north-western Uruguay and throughout the phytogeographic Chaco province in Argentina, Bolivia and Paraguay (Morrone *et al.*, 2000). This species includes sexual diploids ($2n = 2x = 20$) and triploids ($2n = 3x = 30$) (Espinoza and Quarin, 1997; Urbani *et al.*, 2002), and triploids, tetraploids ($2n = 4x = 40$) and hexaploids ($2n = 6x = 60$) that reproduce by pseudogamous aposporous apomixis (Nath *et al.*, 1970; Caponio and Quarin, 1987; Urbani *et al.*, 2002). Genetic analyses of tetraploid intra-specific hybrids of *P. simplex* revealed that apomixis was under monogenic genetic control (Pupilli *et al.*, 2001). Rice molecular markers linked to apomixis in *P. simplex* were also detected (Pupilli *et al.*, 2001).

Sexual tetraploid plants of *P. notatum* and *P. simplex* have never been collected in the wild. All entirely sexual tetraploids of these species have been obtained experimentally through chromosome doubling of sexual diploid cytotypes (Forbes and Burton, 1961; Cáceres *et al.*, 1999). Crosses of induced tetraploid plants (as the female parent) with pollen of a natural apomictic tetraploid may then produce new sexual tetraploids as well as apomictic tetraploids. New sexual tetraploid genotypes might eventually be obtained through self-pollination of induced sexual tetraploids (Martínez *et al.*, 2001), although the induced $4x$ plants usually retain the self-incompatibility system of the original diploids.

The aim of this work was to evaluate the patterns of apomixis transmission in *Paspalum*, in relation to the ploidy levels of gametes, and therefore to determine why apomixis is absent at the diploid level.

MATERIALS AND METHODS

Plant material

Paspalum notatum and *P. simplex* were used for this study. The plant material and its origin are described in Table 1. All plants were cultivated at IBONE, Corrientes, Argentina. Sexual genotypes with different ploidy levels were used as female parents and apomictic triploid genotypes as pollen donors. Sexual diploid and tetraploid genotypes are expected to produce haploid male and female gametes, while triploid genotypes can generate male gametes with different chromosome numbers. Previous studies have showed that triploid genotypes of both species could generate offspring through monoploid ($n = x$), diploid ($n = 2x$) and aneuploid male gametes (E. J. Martínez, unpubl. res.). On the other hand, apomictic triploids of both species form unreduced triploid ($3x$) female gametes in the aposporous embryo sacs.

Breeding scheme for the transmission of apomixis

Sexual diploid ($2n = 2x = 20$) and tetraploid ($2n = 4x = 40$) accessions of *P. notatum* were crossed with an apomictic conspecific triploid ($2n = 3x = 30$) genotype. Otherwise, sexual diploid, triploid and tetraploid accessions of *P. simplex* were crossed with an apomictic triploid plant of the same species. Controlled crosses were made in a fog chamber as described by Burton (1948). Florets from the female parents were emasculated and pollen from the

TABLE 1. *Origin, identification, chromosome number, and method of reproduction of the genotypes used in the present study*

Species and accessions	Ploidy level and chromosome number	Method of reproduction	Origin
P. notatum			
Q4084	$2x = 20$	Sexual	Argentina, Santa Fe, Cayastá. Four genotypes were used
Q3658	$2x = 20$	Sexual	USA, Georgia, Tifton, line #2
Q4160	$2x = 20$	Sexual	USA, Georgia, Tifton, line #9
Q4175	$2x = 20$	Sexual	Argentina, Santa Fe, San Justo, La Criolla
C4-55	$2x = 20$	Sexual	Argentina, Corrientes (experimental origin)
Q3686	$3x = 30$	Apomictic	Argentina, Corrientes, Sauce, Paso Mula
Q4188	$4x = 40$	Sexual	F_1 hybrid between tetraploid strain Q3664 and Q3853
Q4205	$4x = 40$	Sexual	Argentina, Corrientes. Plant obtained by self-pollination of Q3664
Q3664	$4x = 40$	Mainly sexual	USA, Georgia, Tifton (experimental origin)
F1-199	$4x = 40$	Sexual	F_1 hybrid between sexual tetraploid strain Q4188 and apomictic Q4117
P. simplex			
C3-32	$2x = 20$	Sexual	Argentina, Corrientes. Selected plant from the third selection cycle of a breeding project
U36	$3x = 30$	Sexual	Argentina, Chaco. Plant obtained from seed of diploid strain
U45	$3x = 30$	Apomictic	Argentina, Chaco. A natural triploid plant
C1-2	$4x = 40$	Sexual	Argentina, Corrientes. Plant obtained from an induced autotetraploid genotype by self-pollination
C1B2	$4x = 40$	Sexual	Argentina, Corrientes. Plant obtained from an induced autotetraploid genotype by self-pollination

male parent was harvested in glassine bags and dusted on to the stigmas of the emasculated florets. Emasculation was performed after anthesis, with sharp pointed tweezers, when the three anthers of each floret hung indehiscent due to the high humidity inside the chamber.

Apomictic triploid cytotypes were used as male parents instead of tetraploids, because they were able to produce gametes with different chromosome numbers from $n = 10$ (monoploid) up to $n = 20$ (diploid). Since the sexual $2x$ and $4x$ cytotypes only produced haploid reduced gametes, with 10 or 20 chromosomes, respectively, it was possible to estimate the chromosome numbers of the male gametes by chromosome counts in the progeny. Therefore, when an apomictic progeny was detected, the chromosome number of the male gamete that transmitted the trait was estimated. However, it was more difficult to ascertain the chromosome number of the male gametes that gave rise to the progeny from crosses between the sexual $3x$ *P. simplex* (female parent) and the apomictic $3x$ genotype (pollen donor), because either one of the parents could generate gametes with a chromosome range from 10 to 20. However, it can be estimated based on the theory of the endosperm balance number. Johnston *et al.* (1980) postulate that each species has an effective endosperm balance number (EBN) required for normal endosperm development and seed formation. The EBN must be in a 2 : 1 maternal to paternal (m : p) genome ratio. Quarin (1999) demonstrated that the EBN hypothesis is effective in *P. notatum* when sexual plants were used as female parents, but pseudogamous apomictic plants set seed regardless of the relationship between m : p genome input for the endosperm formation. According to this, if a sexual $2x$ is pollinated with an apomictic $3x$, it would be expected to give rise to progeny with $2n = 2x = 20$, because female input for endosperm (two reduced polar nuclei, $x + x$) should be balanced by a ten or near-to-ten chromosome male gamete from the male apomictic triploid. Likewise, the offspring from sexual $4x$ by apomictic $3x$ crosses should mainly be tetraploid. In this case, the sexual tetraploid plant contributes with two reduced polar nuclei ($2x + 2x = 4x$), while the apomictic triploid should provide a diploid or near-diploid sperm ($n = 2x$) for effective endosperm development. It is expected that crosses among two $3x$ plants would produce progeny when a male gamete matches a female one with an equal or very similar chromosome number. Fusion between gametes with different chromosome numbers will produce ratios higher or lower than 2 : 1.

Molecular markers linked to the apomixis locus (apo-locus) and embryological analyses of mature embryo sacs were used to identify the apomictic plants in the progeny. In *P. notatum*, two randomly amplified polymorphic DNA (RAPD) markers completely linked to the apo-locus were used (Martínez *et al.*, 2003). Previous analysis confirmed that an apomictic $3x$ genotype of *P. notatum* used in this study amplified the two RAPD markers linked to the apo-locus (E. J. Martínez, unpubl. res.). Otherwise, a sequence-characterized amplified region (SCAR), which is specific for apomixis in *P. simplex* (Calderini *et al.*, 2006), was employed to identify the apomictic progeny of this species, and confirmed by embryological analyses. SCAR markers were obtained according to Paran and Michelmore (1993).

Hybrid progeny identification

Because some female parents produced a few seeds under self-pollination conditions, and because also the emasculation procedure might admit some minimal degree of self-pollination, the diploid offspring derived from the $2x \times 3x$ crosses and the tetraploid descendants

from the $4x \times 3x$ crosses were controlled to guarantee their hybrid origin. Hybridity was examined by progeny tests with molecular markers as it was described in Acuña *et al.* (2004).

Chromosome numbers and embryological analyses

Chromosome counts were performed for each individual plant that amplified the molecular markers linked to the apo-locus in order to determine the ploidy or chromosome number of the male gamete that produced that plant. In addition, several plants from each cross, that did not amplify such markers, were also counted to assess the chromosome number of gametes that failed to transmit apospory. For chromosome counts, root tips were placed in a saturated solution of bromonaphtalene for 2 h, hydrolysed in 1 N HCl for 10 min at 60°C, stained with Fuelgen reagent, squashed in a drop of 1% aceto-orcein, and observed with a light transmission microscope. Five to ten metaphase mitoses with well-defined spread chromosomes were scored for each individual analysed.

Embryological analyses were based on mature embryo sac observations, carried out only on those plants that amplified the markers associated with apomixis, as described by Martinez *et al.* (2001).

RESULTS

Transmission pattern of apomixis in Paspalum notatum

Intraspecific crosses were made between eight diploid genotypes of *Paspalum notatum* as female parents and one apomictic triploid genotype as the male parent. Thirty-seven individuals were recovered from a total of 11 532 pollinated florets. The reproductive efficiency (percentage of plants obtained from total number of pollinated florets) was very low in all combinations with a general mean of 0·32% (Table 2). Chromosome counts confirmed 34 diploids ($2n = 2x = 20$), one triploid ($2n = 3x = 30$) and two aneuploids ($2n = 22$ and 29). Then, all diploid descendants were evaluated with several RAPD markers that proved to be specific for the triploid parent in order to rule out self-pollination and verify their hybridity. All thirty-four diploid progeny amplified specific bands derived from the triploid parent, indicating their hybrid origin. The number of male-specific bands observed in each diploid descendant varied from a minimum of three to a maximum of nine. It was determined that monoploid gametes ($n = x = 10$) from the apomictic triploid parent contributed to the formation of $2x$ progeny. None of these diploid hybrids amplified the RAPD markers linked to the apo-locus (Table 2).

The individual cross concerning the diploid genotype Q4084 #2 produced eight diploid, one triploid and one hypotriploid ($2n = 29$) descendants. Both the triploid and the near-triploid progeny amplified RAPD markers linked to the apo-locus (Table 2), and showed a high percentage of mature ovules carrying one or more aposporous embryo sacs (data not shown). These results substantiated the transmission of apospory through diploid ($2x$) or hypodiploid ($2x - 1$) gametes.

Intraspecific crosses were also made between four sexual tetraploid genotypes of *P. notatum* and the apomictic triploid plant (Table 2). From a total of 11 493 pollinated florets, 154 individuals were recovered. This resulted in an average reproductive efficiency of 1·34% (Table 2). Chromosome counts ranged from $2n = 30$ to 42. However, most plants

TABLE 2. *Crosses performed to determine the type of apomixis transmission between sexual diploid and tetraploid accessions, and an apomictic triploid genotype of* Paspalum notatum

Crosses	Crossed florets (no.)	Plants obtained (no.)	Reproductive efficiency (%)*	Number of plants with $2n$													Plants with RAPD markers (no.)
				20	22	29	30	31	32	36	37	38	39	40	41	42	
$2x \times 3x$																	
Q4084 #1 × Q3686	743	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Q4084 #2 × Q3686	2904	10	0·34	8	–	1	1	–	–	–	–	–	–	–	–	–	2†
Q4084 #8 × Q3686	1556	7	0·45	7	–	–	–	–	–	–	–	–	–	–	–	–	0
Q4084 #9 × Q3686	395	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	0
Q3658 × Q3686	398	0	0	–	–	–	–	–	–	–	–	–	–	–	–	–	0
Q4160 × Q3686	2976	9	0·3	9	–	–	–	–	–	–	–	–	–	–	–	–	0
Q4175 × Q3686	1264	7	0·55	6	1	–	–	–	–	–	–	–	–	–	–	–	0
C4-55 × Q3686	1296	4	0·31	4	–	–	–	–	–	–	–	–	–	–	–	–	0
Total	11532	37	0·32	34	1	1	1	–	–	–	–	–	–	–	–	–	2
$4x \times 3x$																	
Q4188 × Q3686	2038	38	1·86	–	–	–	–	1	1	–	–	–	6	26	2	1	0
Q4205 × Q3686	4585	40	0·87	–	–	–	–	–	1	1	1	1	4	31	1	–	1‡
$F_1$199 × Q3686	2665	29	1·09	–	–	–	–	–	–	–	–	1	–	29	–	–	0
Q3664 × Q3686	2205	47	2·13	–	–	–	1	–	–	1	4	–	10	30	1	–	0
Total	11493	154	1·34	–	–	–	1	1	2	2	5	2	20	116	4	1	1

* Reproductive efficiency: percentage of plants obtained from the total number of pollinated florets.
† One of them with $2n = 29$ and another with $2n = 30$.
‡ Plant with $2n = 39$.

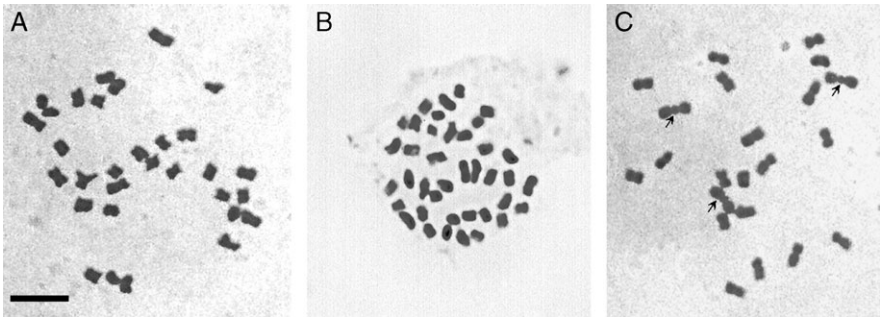

FIG. 1. Mitotic metaphase chromosomes in some progeny from intraspecific crosses of *Paspalum simplex* and *P. notatum*: (A) progeny plant with $2n = 31$ chromosomes from sexual tetraploid (Q4188) × apomictic triploid (Q3686) cross of *P. notatum*; (B) progeny plant with $2n = 39$ chromosomes from sexual tetraploid (Q3664) × apomictic triploid (Q3686) cross of *P. notatum*; (C) progeny plant with $2n = 22$ chromosomes from sexual triploid (U36) × apomictic triploid (U45) cross of *P. simplex*. The arrows indicate three homologous chromosomes with satellites. The basic chromosome set of *P. simplex* has a characteristic big chromosome bearing a second constriction with a large satellite. Scale bar = 5 μm.

(75%) had $2n = 40$ chromosomes and, furthermore, 91% of the progeny ranged from $2n = 39$ to $2n = 41$. Other aneuploid numbers were less frequently produced, and only one plant had $2n = 30$. Several molecular markers (from three to ten) specific of the male triploid parent were generated in each of the tetraploid ($2n = 40$) progeny by RAPD procedures as a verification of their hybrid origin. Chromosomes in mitotic metaphase of a progeny plant with $2n = 31$ and another with $2n = 39$ from sexual $4x$ × apomictic $3x$ crosses are illustrated in Fig. 1A and B, respectively. These results indicate a very low recovery of plants from a diploid egg cell ($n = 2x$) and a monoploid sperm nucleus ($n = x$). Most descendants originated from the normal $2x$ egg cell and a male gamete carrying $n = 20$ or close to 20. Results indicated that in a few cases, female aneuploid gametes with chromosome numbers close to $n = 20$ (higher or lower than 20) might give rise to some descendants. Although 91% of the progeny originated from diploid or near-diploid gametes of the apomictic triploid parent, only one plant showed the molecular markers linked to the apo-locus. This individual had $2n = 39$ and arose from the sexual $4x$ plant Q4205 and the male $3x$ parent (Table 2). A total of 153 ovules of this particular progeny were analysed during two flowering periods, and only two ovules showed aposporous embryo sac development. The behaviour indicated that it was a facultative apomictic plant, with a low expression of the trait, and that it was transmitted through a hypodiploid male gamete ($n = 2x - 1 = 19$).

Transmission pattern of apomixis in Paspalum simplex

The transmission of apomixis in *Paspalum simplex* was analysed by crossing a natural apomictic triploid with sexual females differing in ploidy: one diploid, one triploid and two tetraploids used as female parents. One hundred and eighteen plants were recovered when a $2x$ genotype was used as the female parent (Table 3). The reproductive efficiency was 9·7% from a total of 1210 pollinated florets. From the 118 plants recovered, none amplified the SCAR marker linked to the apo-locus (Table 3 and Fig. 2A). Therefore, chromosome counts were only carried out on a random sample of 28 plants, which were all diploids ($2n = 20$). The hybrid origin of the whole

TABLE 3. *Crosses performed to determine the feasibility of transmitting apomixis and the method of transmission from an apomictic triploid genotype of* Paspalum simplex *to sexual genotypes (diploids, triploids and tetraploids) of the same species*

Crosses	Pollinated florets (no.)	Plants obtained (no.)	Reproductive efficiency (%)	Number of plants with 2n															Plants with SCAR marker (no.)
				20	21	22	23	24	25	26	27	28	30	32	37	38	39	40	
Sexual $2x$ × apomictic $3x$																			
C3-32 × U45	1210	118*	9·7	28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
Sexual $3x$ × apomictic $3x$																			
U36 × U45	1783	64†	3·6	4	10	8	9	5	1	1	1	3	2	2	1	1	1	0	2‡
Sexual $4x$ × apomictic $3x$																			
C1B2 × U45	817	14	1·7	–	–	–	–	–	–	–	–	–	–	–	1	2	4	7	3§
C1-2 × U45	1139	23	2·0	–	–	–	–	–	–	–	–	–	–	–	–	–	7	16	0

* Chromosome counting was only performed on 28 randomly selected plants.
† Chromosome numbers were performed on 49 plants because the remaining 15 plants died at seedling stage.
‡ One of them with $2n = 27$ and another with $2n = 32$.
§ All plants with $2n = 39$.

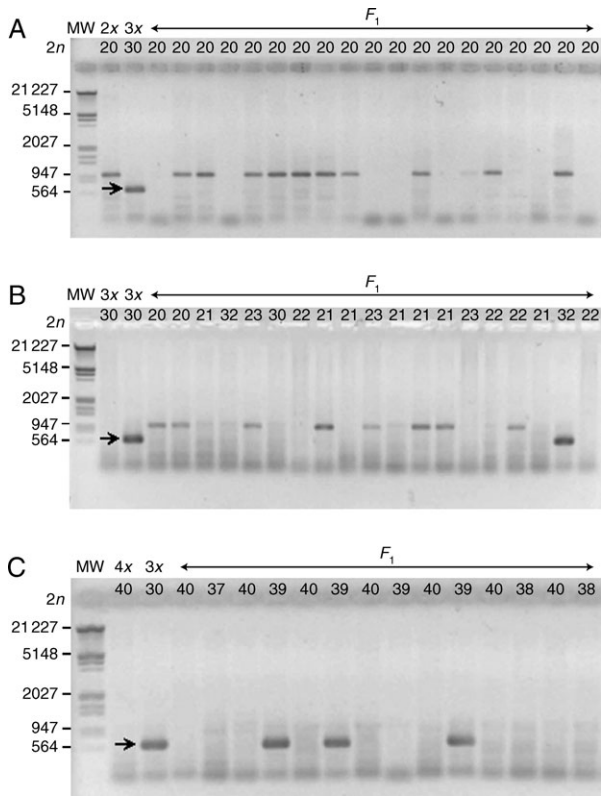

FIG. 2. Apomixis-specific SCAR in three progeny of *Paspalum simplex*: (A) SCAR amplification pattern for the sexual diploid mother C3–32 (2*x*), the apomictic triploid male U45 (3*x*) and 19 diploid F_1 progeny; (B) SCAR amplification pattern for the sexual triploid mother U36, the apomictic triploid male U45, and 19 F_1 progeny with varying chromosome numbers from $2n = 20$ to $2n = 32$; (C) SCAR amplification pattern for the sexual tetraploid mother C1B2, the apomictic triploid male U45 and 14 F_1 offspring as a sample of variability in chromosome number from $2n = 37$ to $2n = 40$. The arrows point to the apomixis-specific fragment of 650 bp. MW = molecular weight marker (lambda *Eco*RI/*Hin*dIII) expressed in base pairs.

diploid offspring was verified by progeny tests with molecular markers specific for the male triploid parent. When the sexual triploid U36 was crossed with the apomictic triploid U45, sixty-four individuals were recovered (Table 3). Chromosome counts could only be performed on 49 plants because the remnant seedlings died. In this cross a wide range of chromosome numbers was observed (Table 3), with 73% of the progeny analysed varying from 20 to 24. Chromosomes of an aneuploid plant with $2n = 22$ are illustrated in Fig. 1C. Two plants amplified the SCAR marker linked to apo-locus and showed aposporous embryo sacs. One of them had 27 chromosomes and the other 32 (Table 3). SCAR amplification patterns for some of the hybrids produced from sexual triploid × apomictic triploid are shown in Fig. 2B.

Two sexual 4*x* accessions (C1-2 and C1B2) were crossed with the apomictic 3*x* plant. A total of 37 hybrids were obtained, 14 of them from C1B2 × U45 and 23 from C1-2 × U45 crosses (Table 3). Plants with 37, 38, 39 and 40 chromosomes were obtained. Most of them, however, had 39 and 40 chromosomes. All plants with $2n = 40$ were confirmed to have originated by hybridization and not by self-pollination. From three to six specific markers from the triploid U45 male parent were detected in the tetraploid descendants. Three plants with $2n = 39$ amplified the SCAR marker (Table 3 and Fig. 2C), and congruently these three exhibited ovules with aposporous embryo sacs, confirming their apomictic reproduction mode (data not shown). All apomictic progenies were generated from C1B2 × U45 crosses (Table 3).

DISCUSSION

The transmission of aposporous apomixis in *Paspalum notatum* and *P. simplex* was studied with the purpose of determining the mechanism responsible for its absence or effectiveness in diploid forms. In *Paspalum*, all known apomictic species are polyploid. However, most of these apomictic polyploids, especially tetraploids, contain conspecific sexual diploid counterparts. In several species of *Paspalum*, these diploid cytotypes have shown occasional aposporous embryo sacs in their ovules (Quarin *et al.*, 1982, 2001; Quarin and Norrmann, 1987; Norrmann *et al.*, 1989). Similar results were observed in the tropical grass *Brachiaria decumbens* (Naumova *et al.*, 1999). According to Naumova *et al.* (1999), the diploid plant of *B. decumbens* was a facultative sexual plant, which probably arose as a dihaploid plant from a natural apomictic tetraploid population. By contrast, Quarin *et al.* (2001) reported that diploid plants of *Paspalum*, with the capacity to form occasional aposporous embryo sacs, have not arisen as dihaploids, but rather they constitute natural diploid populations. Whether these eventual aposporous sacs are able to generate maternal progeny is an issue that has not yet been proved.

The strategy used in this work permitted testing of the hypotheses proposed by Nogler (1982) and Mogie (1988) about the absence of apomixis at the diploid level in *Paspalum*. To achieve this, an attempt was made to produce hybrids, using pollen (from $n = x$ to $n = 2x$) of apomictic triploids, for crosses with sexual cytotypes of the same species (2*x*, 3*x*, 4*x*).

In both *Paspalum* species, crosses between sexual diploid and apomictic triploid pollen donors generated almost exclusively diploids. Only one triploid and two aneuploids were obtained from 2*x* × 3*x* crosses of *P. notatum*. This indicates that reduced eggs ($x = x = 10$) from diploid mother plants were fertilized by monoploid gametes ($n = x = 10$) from the male parent. None of these diploid plants, however, amplified the molecular markers specific for apomixis. This indicates that monoploid male gametes, carrying the apomixis trait, were not transmitted to the progeny, at least when the progeny was diploid. Intriguingly, in these 2*x* × 3*x* crosses of *P. notatum* the 3*x* and the near-3*x* apomictic plants were both generated from a single cross in which the female 2*x* parent was Q4084 #2. According to previous studies (Quarin *et al.*, 2001), this plant (identified as R2) has revealed a capacity for very occasional aposporous embryo sac development besides the regular meiotic sac. These previous results leave room for speculation about the gametes that gave rise to both the triploid and the hypotriploid ($2n = 29$)

plants. They might be formed from a non-reduced female gamete and monoploid ($n = 10$) or hypomonoploid ($n = 9$) male gametes. If this was the case, apomixis might be transmitted by either male ($n = x$) or female ($2n = 2x$) gametes, but only expressed due to the polyploid condition of the progeny. Although theoretically possible, the likelihood seems to be quite limited since the $2x$ plant (the female parent) usually produces monoploid gametes, and the $3x$ plant (the male apomictic parent) can form gametes with a wide range of chromosome numbers from monoploid ($n = 10$) to diploid ($n = 20$) and all possible aneuploids from $n = 11$ to $n = 19$. Consequently, the $3x$ and the hypo-$3x$ plants were most likely recovered following fertilization of a monoploid egg with a diploid male gamete ($n = 2x = 20$ or $n = 2x - 1 = 19$).

Because male meiosis in apomictic triploids may produce an array of gametes with different chromosome numbers, from $n = 10$ to $n = 20$, it could be expected that a higher number of triploids or aneuploids would be recovered in $2x \times 3x$ crosses. However, most descendants were formed by male gametes with $n = 10$. This means that male gametes were effective when their chromosome number balanced the chromosome number of the female gametes. Equivalent results were observed when sexual tetraploid plants were crossed with apomictic triploids. Most of the progeny recovered were tetraploids or with chromosome numbers very close to 40. In general, the progeny arose when gametes with identical or very close chromosome numbers fused. This reveals that most triploid ($10 + 20$ or $20 + 10$) or aneuploid products from interploidy crosses were not viable. This poor recovery could be due to a deviation from the normal 2 : 1 maternal : paternal genome contribution for endosperm development. Proper development of the endosperm is essential for growth of the embryo and production of viable seed. Most sexually reproducing species need a delicate 2 : 1 maternal to paternal genome ratio for the development of normal endosperm and seed production (Johnston *et al.*, 1980). In diploid progeny recovered from $2x \times 3x$ crosses, the embryo would be diploid and the endosperm triploid, receiving two maternal genomes and one paternal genome (2 m : 1p). Meanwhile, in triploid progeny the embryo would be triploid and the endosperm tetraploid (2 m : 2p). Something similar occurred in the offspring recovered from $4x \times 3x$ crosses. Most of them were tetraploids, where the endosperm would be hexaploid ($6x$), receiving four maternal and two paternal genomes (2 m : 1p ratio). Ratios higher or lower than 2 : 1 resulted in very low seed set. However, pseudogamous apomictic strains of *P. notatum* appear to be more flexible because endosperm development occurs regardless of the ploidy level of the pollen donor (Quarin, 1999). A poor recovery of triploid progeny plants has also been observed in crosses between sexual diploid and apomictic tetraploid genotypes of *P. notatum* (Burton and Hanna, 1992). Similar results were obtained when conspecific crosses between $2x$ sexual and $4x$ apomictic were carried out in five species of *Paspalum* (Norrmann *et al.*, 1994). Triploid intraspecific hybrids were recovered at very low frequencies in *P. intermedium* and *P. brunneum*, but no $3x$ progenies were obtained in *P. almum*, *P. quadrifarium* and *P. rufum* (Norrmann *et al.*, 1994).

In *P. simplex*, the triploid plants are rare members of an agamic complex that most likely originated by autoploidy (Urbani *et al.*, 2002). Gametes of these triploids are theoretically expected to have any chromosome number from $n = x = 10$ (monoploid) to $n = 2x = 20$ (diploid). In fact, the present results from $2x \times 3x$, $3x \times 3x$, and $4x \times 3x$ crosses suggested that triploid plants produced a wide array of gametes from $n = 10$ to $n = 20$. However, the effectiveness of these gametes when acting from the male side depended on the chromosome number of the female gamete: when the egg cell was monoploid ($n = 10$), the successful male gamete was also monoploid, giving rise to $2x$ hybrids ($2n = 20$). When the female parent was a tetraploid with expected $n = 2x = 20$ egg cells, most hybrids originated from diploid or near-diploid male gametes ($n = 20$ or $n = 19$). The chromosome number of the egg cell was decisive in selecting the chromosome number of the effective male gamete. This selection could act through a precise operation of the endosperm balance number system, requiring 2 : 1 female:male genome input for endosperm development. Thus, the aneuploid hybrids recovered from $3x \times 3x$ crosses originated most likely from the fusion of an egg cell and a sperm nucleus with identical or very similar chromosome numbers. Consequently, the two $3x \times 3x$ hybrids ($2n = 27$ and $2n = 32$) that amplified the SCAR marker linked to the apo-locus originated from $14 + 13$ (or $13 + 14$) and $16 + 16$ gametes, respectively. Similarly, the three hybrids ($2n = 39$) that amplified the SCAR when the female parent was $4x$ were probably formed by hypodiploid sperm nuclei ($n = 2x - 1 = 19$) matching diploid female gametes, although inverse fusion, female $n = 19$ and male $n = 20$, could not be excluded from a theoretical standpoint.

The present results indicate that many of the monoploid male gametes from the triploid parent were functional and their products viable. However, no diploid progeny showed the molecular markers completely linked to apomixis, indicating that male monoploid gametes carrying the apomixis locus were not transmitted to the diploid progeny. In contrast, when a sexual tetraploid was used as a female parent, offspring typically arose from diploid or near-diploid gametes of the apomictic triploid parent. Thus, formation of polyploid (triploid) progeny by means of a monoploid male gamete does not occur, concluding that apomixis would not segregate to polyploid progeny through a monoploid gamete. Only one triploid plant arose from a monoploid male gamete of the triploid parent and it was sexual; most likely the monoploid male gamete was lacking the factor for apomixis. In *Paspalum*, therefore, selection of the male gametes is biased by the chromosome number of the female gamete. This behaviour would be strongly associated with the requirement of an endosperm genomic balance.

Interestingly, the aneuploid apomictic progenies ($2n = 27$ and $2n = 32$) from $3x \times 3x$ crosses of *P. simplex* suggest that apomixis could also be transmitted by gametes carrying chromosome numbers (13, 14 or 16) considerably lower than the diploid condition. Since the parents

were autotriploids, the transmission of apomixis would require disomy rather than diploidy in the transmitting gamete.

No recovery of apomictic diploids through mediation of monoploid gametes could be explained by a linked lethal system as described by Nogler (1982). However, it was not possible to verify that monoploid gametes did not transmit the trait to polyploid progeny. Nogler's hypothesis states that gametes carrying the apomixis factor should be heterozygous because the homozygous condition is lethal. Therefore, monoploid gametes carrying the control of apomixis would be unviable. The present results cannot be used to support Nogler's hypothesis if it is not possible to enlarge the polyploid (triploid) population generated by means of monoploid male gametes. However, the present results do favour the theory that apospory, at least in 4*x P. notatum*, is controlled by a single dominant gene with a pleiotropic lethal effect with incomplete penetrance (Martínez *et al.*, 2001).

The present results do not fit the hypothesis proposed by Mogie (1988). According to this hypothesis, apomixis arises when the ratio of apomictic *vs*. sexual alleles is $>0{\cdot}5$, meaning that a dose of genetic factor(s) for apomixis should be present in diploids. Therefore, molecular markers completely linked to the apomictic trait should be detected. In turn, markers should also be detected in sexual polyploid plants carrying only one copy of the allele conferring apomixis. None of these markers were amplified in the sexual progeny generated in this work. Bicknell *et al.* (2000) observed a different apomixis transmission pattern in *Hieracium* species with aposporous apomixis. Diploid and tetraploid sexual genotypes of *H. pilosella* were crossed with apomictic triploids of *H. piloselloides*. When diploid sexual plants were used as the mother plant, very few diploid progeny were recovered. Most of them were triploids or aneuploids and apomixis was only transmitted by diploid gametes. However, when sexual tetraploids were crossed with apomictic triploids, most progeny were triploid and tetraploid. Apomixis segregated among both the triploid and tetraploid progeny as a monogenic dominant trait. This indicates that both monoploid and diploid gametes were able to transmit the apomixis trait. Non-recovery of diploid apomictic progeny was a consequence of selection against diploid hybrids, acting after fertilization, rather than selection at the gamete level (Bicknell *et al.*, 2000).

In conclusion, the present study showed that the apomixis factor(s) in *Paspalum* is not transmitted by exact monoploid gametes. This finding explains why apomictic diploids cannot be generated by hybridization. There was a strong distorted segregation of the apomixis trait when it was transmitted through diploid or higher-than-monoploid gametes. Incomplete penetrance of the lethal effect associated with apomixis, or alternatively, of some factor linked to the apo-locus could be the mechanism causing distorted transmission of the genomic region responsible for apomixis in *Paspalum*. Future analyses should be carried out to determine why some diploid species of *Paspalum* show some elements of apomictic reproduction, as the occasional formation of aposporous embryo sacs, and if these sacs are able to generate maternal progeny. In addition it should be determined if this ability by diploids is associated with the generation of apomictic polyploids after colchicine induction from sexual diploids.

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