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Apospory followed by sterility in a hypotriploid hybrid (2x X 4x) of *Paspalum*

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Abstract — A self-incompatible diploid plant (2n = 2x = 20) of *Paspalum limbatum* grown in a field nursery surrounded by tetraploid accessions of several *Paspalum* species produced a hypotriploid descendant (2n-1 = 3x = 29). Molecular fingerprinting by RAPD markers indicated that an apomictic 4x accession of *P. guenoarum* was the pollen parent. Meiotic chromosome pairing in the hypotriploid hybrid averaged 16.3 univalents (range 9-29), 5.4 bivalents (0-9), and 0.7 trivalents (0-3). Since *P. guenoarum* has an autopolyploid or segmental allopolyploid origin, the bivalent associations in the hypotriploid hybrid could be ascribed to an autosyndetic pairing between the two chromosome sets contributed by the male parent. The trivalent chromosome associations suggested some degree of homology between the chromosomes of *P. limbatum* and *P. guenoarum*. Embryological analysis of the hypotriploid hybrid showed complete abortion of the megaspore mother cell before or during the first meiotic division. One to several aposporous embryo sacs developed from somatic nucellar cells in over 97% of the ovules analysed. The plant was 100% male sterile and seed sterility reached 99.9%, despite hand-pollination with several pollen sources. Thus, apospory was fully inherited from the apomictic 4x male parent, but the trait did not prevent seed sterility.

Key words: apomixis, apospory, fertility, *Paspalum limbatum*, *Plicatula.*

INTRODUCTION

Paspalum is a grass genus characterized by a variable genetic system: with a few exceptions, the basic chromosome number is x = 10 and polyploidy is the rule. Approximately 80% of the species so far investigated are polyploids with a predominance of tetraploids. Most polyploid *Paspalum* species reproduce by apomixis and usually have co-specific, sexual, self-incompatible diploid races (QUARIN 1992). Apospory is the most common type of apomixis in *Paspalum.*

Paspalum limbatum Henrard is a small representative of the group *Plicatula* and is found in eastern Bolivia, Paraguay, and northeastern Argentina. It is characterized by small spikelets (1.5-2 mm long), slender culms, and narrow leaf blades. This species is diploid (NORRMANN *et al.* 1994), sexual, and allogamous due to self-incompatibility (ESPINOZA *et al.* 2001).

The objectives of this research were: 1) to determine, through the assistance of molecular markers, the possible pollen parent of a spontaneous hypotriploid hybrid descended from a diploid plant of *P. limbatum*; 2) to estimate the genomic relationship between parental species through the analysis of meiotic chromosome pairing in the hypotriploid hybrid; 3) to establish the reproductive mode of the hypotriploid through an embryological survey and analysis of fertility.

MATERIALS AND METHODS

Plant material - A single diploid (2n = 2x = 20) plant of *P. limbatum* was established in a *Paspalum* nursery at IBONE, Corrientes, Argentina, from seed collected in Bolivia, Dept. Santa Cruz, 10 km S of Concepción on the road to Lomerío, 16°08'S, 62°00'W, and was provided by Dr. Timothy J. Killeen, Missouri Botanical Garden, collection # 2453 (herbarium voucher at CTES). All inflorescences formed in 1994 were harvested at maturity following open-pollination. Only two seedlings were recovered, and these plants were grown in pots in a greenhouse. One plant resembled the mother plant and had 2n = 2x = 20 chromosomes. It most likely originated from a rare self-fertilization event. The second plant was an off-type and showed a hypotriploid level (2n = 3x -1 = 29). Cuttings of the off-type were used to estab-

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lish several clones in pots and in the field. A herbarium voucher (Quarin # 4218) was deposited in the CTES herbarium.

There were several *Paspalum* species in the nursery whose flowering cycles overlapped the flowering period of the diploid *P. limbatum* plant. All of them were tetraploid apomictic species of the *Plicatula* group and had been established from seed of the following species and accessions: *P. lenticulare* Kunth (TK2270, TK2322, TK2396, TK2417); *P. guenoarum* Arechav. (TK2390, TK2394); *P. kempffii* Killeen (TK2272), *P. macedoi* Swallen (TK2323); *P. plicatulum* Michx. (TK2455), *P. plicatulum* var. *intumescens* Döll (Q4087); and *P. atratum* Swallen (Q4096). A single plant of each one was in the nursery surrounding the diploid plant of *P. limbatum* (TK2453) in 1994 when the mature spikelets of this plant were harvested.

DNA extraction and RAPD procedure - Genomic DNA was extracted from young leaves according to MARTÍNEZ *et al.* (2003). Randomly amplified polymorphic DNA markers (RAPDs) were used to identify the pollen donor parent that produced the hypotriploid hybrid (Q4218) of *P. limbatum*. Because RAPDs are dominant markers, any DNA fragment that amplifies in the Q4218 hybrid but not in the female parent, comes from the male parent. The screening of enough primers that develop specific bands in Q4218 would allow the identification of the candidate pollen parent from the tetraploid species (2n= 4x = 40) that were cultivated in the *Paspalum* nursery in 1994, and that flowered simultaneously with the diploid mother plant *P. limbatum*. Forty random decamer primers (set 5) from the RAPD Primer Synthesis Project of the University of British Columbia (UBC) were screened to find polymorphisms between the female parent and the Q4218 hybrid. The reproducibility of the results was evaluated by running PCR reactions twice for the same DNA on each individual analysed. PCR reactions and band detection were similar to those described by MARTÍNEZ *et al.* (2003).

Cytological, embryological and fertility analysis - Methods used to study mitosis, meiosis, megasporogenesis, embryo sac development, and pollen stainability are similar to those previously reported by QUARIN and HANNA (1980). Two techniques were used to determine female fertility: a) percent seed set b) pollen germination and pollen tube growth, which was observed according to the technique of KHO and BAËR (1968).

RESULTS

Identification of the male parent - A total of 40 random primers were screened in order to identify specific bands in the hypotriploid hybrid Q4218, which were absent in the female diploid parent TK2453. The screening led to the identification of 35 primers that produced good amplification signals with a mean of 5 bands each. Five primers amplified 6 bands that were specific to the hypotriploid hybrid and were absent in the female parent. Four of them amplified one specific band and the fifth amplified two bands (Table 1).

Genomic DNAs of the six tetraploid *Paspalum* species were analysed with the selected RAPD primers. None of the bands specific to the hypotriploid hybrid were observed in *P. kempffii*, *P. macedoi*, or the four *P. lenticulare* accessions. Only two of the specific bands were generated in TK2394 (*P. guenoarum*) and TK2455 (*P. plicatulum*), while *P. plicatulum intumescens* and *P. atratum* accessions shared three bands that were specific to the hypotriploid hybrid (Table 1). Amplifications of genomic DNA from TK2390 (*P. guenoarum*) showed the six bands

Table 1 — Male parent identification of a hypotriploid hybrid progeny from 2x *Paspalum limbatum*. Seven taxa (concerning eleven accessions) were screened for RAPD markers that were present in the 3x hybrid and absent in the female 2x parent. The informative markers were generated by five decamer primers of the University of British Columbia series (UBC). Only the accession TK2390 of *P. guenoarum* showed amplification of all six dominant markers specific to the hypotriploid hybrid.

Primers generating specific bands in the hypotriploid hybrid Q4218	*P. lenticulare*				*P. atratum*	*P. guenoarum*		*P. kempffii*	*P. plicatulum*	*P. plicatulum var. intumescens*	*P. macedoi*
	TK2270	TK2322	TK2396	TK2417	Q4096	TK2390	TK2394	TK2272	TK2455	Q4087	TK2323
UBC403	-	-	-	-	-	+	-	-	+	-	-
UBC424	-	-	-	-	+	+	+	-	-	+	-
UBC446	-	-	-	-	-	+	-	-	-	-	-
UBC460 (1)	-	-	-	-	+	+	-	-	+	+	-
UBC460 (2)	-	-	-	-	+	+	+	-	-	+	-
UBC 466	-	-	-	-	-	+	-	-	-	-	-

previously found to be specific to the hypotriploid (Table 1). Examples of DNA fingerprints obtained with two different primers are illustrated in Figure 1. Since the RAPDs are dominant markers, only accession TK2390 could be regarded as the pollen parent of Q4218. Only TK2390 carries all six diagnostic RAPD markers, among the 11 *Paspalum* accessions that flowered simultaneously with the *P. limbatum* parent (TK2453) in 1994.

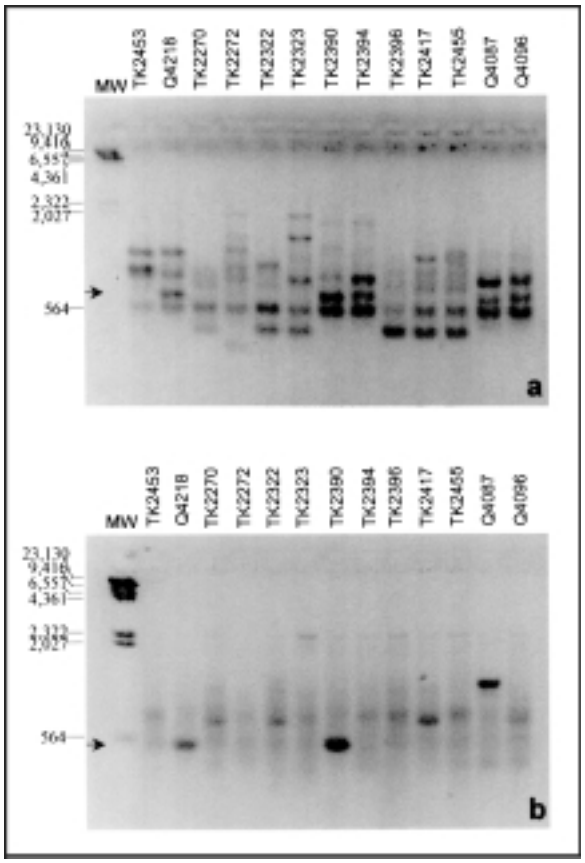

Fig. 1 — **a-b** Fingerprinting to determine the paternity of a hypotriploid hybrid (Q4218) originated from a diploid *Paspalum limbatum*. Amplification by PCR and electrophoretic separation on a 2% agarose gel of genomic DNA from all tetraploid individuals suspected of paternity. **a** - Amplification pattern of UBC424 primer. The arrow indicates the specific band of the hypotriploid hybrid shared by TK2390, TK2394, Q4087, and Q4096. **b** - Amplification pattern of UBC466 primer. The arrow shows a specific fragment of the hypotriploid hybrid amplified only by accession TK2390 of *P. guenoarum*. MW = molecular weight marker (Lambda *Hin*d III from Promega).

Cytology of the hybrid - Chromosome counts in root tips showed that the target plant Q4218 was a hypotriploid with 2n = 3x -1 = 29. Mean chromosome configurations observed in a total of 64 pollen mother cells at diakinesis and metaphase I of meiosis were: 16.3 univalents, 5.4 bivalents, and 0.7 trivalents, with a wide range of univalents (9-29), and bivalents (0-9), and some occasional trivalents (0-3). Lagging chromosomes and micronuclei were always observed at anaphase I and telophase I, respectively, as well as during anaphase II and telophase II.

Embryology - The megaspore mother cell (MMC) of 3x hybrid developed from a subepidermal cell of the nucellus. It is characterized by its large and elongate size, and is surrounded by a sheath of callose. The callose layer was thick around the micropylar end of the MMC and progressively thinner toward the chalazal end. The MMC completed its development before the growing inner integument extended beyond the middle of the ovule. At approximately this stage, the MMC deteriorated in a large proportion (68%) of the observed ovules. In the remaining young ovules, the first meiotic division occurred and a dyad was formed. However, the second division did not occur and, the dyads always degenerated. Immediately after or even before the deterioration of the MMC or the dyad, one or usually several nucellar cells enlarged, developed denser staining cytoplasm, and developed conspicuous nucleoli. These were the initial cells of the aposporous embryo sacs. Two or three incomplete rounds of mitotic divisions gave rise to aposporous embryo sacs that usually had 3 or 4 cells: the egg cell, one or two synergids, and a large central cell with two polar nuclei. The absence of antipodal cells was characteristic of these aposporous sacs (Figure 2). The number, orientation, and degree of development of the aposporous sacs were variable at maturity of the ovule. Over 97% of the mature ovules showed one to several aposporous megagametophytes. Usually, the embryo sac located in front of the micropyle was the largest one. Abortion or lack of embryo sac development occurred occasionally and less than 3% of the ovules lacked an embryo sac at anthesis.

Fertility - Pollen grains of fully developed anthers appeared empty and did not stain when treated in a 2% I_2KI solution, indicating absence of starch. Some spikelets failed to anthese. When blooming, the anthers did not dehisce.

Pollen from diploid and tetraploid species of the *Plicatula* group, including the diploid mother plant (*P. limbatum*) and accession TK2390 (*P. guenoarum*), were dusted on the triploid inflorescences at anthesis. Approximately 15,000 pollinated spikelets from several triploid inflorescences were harvested at maturity, and only 15 seeds were recovered. Six grains were sown in sterilized soil and none of them germinated. Caryopses of the remaining 9 spikelets were dissected from spikelets and cultivated *in vitro*, and two of these germinated. Chromosome counts in root tips indicated that both seedlings had the same chromosome number as the maternal hypotriploid plant (2n-1 = 3x = 29).

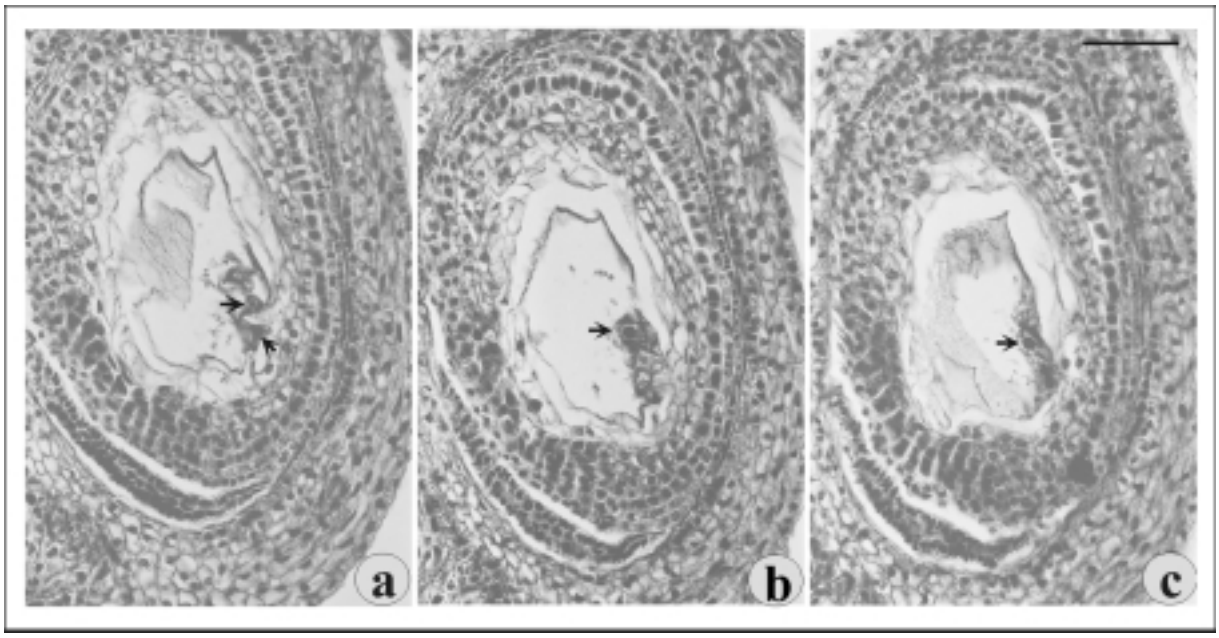

Fig. 2 — **A-C** Mature ovule of the triploid hybrid Q4218. Three consecutive sections of the same ovule with a single aposporous embryo sac. **A** - The egg cell (horizontal arrow) and the degenerated synergid (oblique arrow). **B** - One polar nucleus of the central cell. **C** -The second polar nucleus of the central cell. Note the characteristic absence of antipodal cells. Bar = 50 µm.

Studies through the inflorescence technique (unpublished data) showed that pollen grains from diploid and tetraploid species of *Plicatula* group germinated. Pollen tubes penetrated the stigma papillae of the hypotriploid but then failed to penetrate the style.

DISCUSSION

The bivalent chromosome associations observed in the hypotriploid Q4218 might indicate some degree of homology between the chromosome set of *P. limbatum* and one chromosome set from the male parent. However, the high number of quadrivalent chromosome associations observed in TK2390 (*P. guenoarum*) suggested that it originated by autopolyploidy (ESPINOZA *et al.* 2001). Therefore, bivalents and trivalents may also suggest autosyndetic pairing between the two chromosome sets contributed by the male parent (bivalents), and homology with some chromosomes of *P. limbatum* (trivalents).

Aposporous apomixis in angiosperms at least depends upon three main processes: 1) circumvention of meiosis in the megasporocyte, 2) development of female gametophytes from somatic cells, and 3) embryo development without fertilization (parthenogenesis). In addition, pseudogamy is a prerequisite for endosperm formation in many aposporous grass species. However, circumvention of meiosis is not a sine-qua-non for the second and third processes. Simultaneous development of embryo sacs of both meiotic and aposporous origin has been observed in the same inflorescence and even in the same ovule in several species of *Paspalum* (QUARIN and HANNA 1980; NORRMANN *et al.* 1989; BURSON 1997). On the other hand, it has been suggested that parthenogenesis is a pleiotropic effect of the gene controlling unreduced embryo sac formation (SAVIDAN 2000). Our results indicate that the hypotriploid plant Q4218 has not inherited from the female parent the ability for sexual reproduction or meiosis. Q4218 was severely disturbed due to the odd ploidy level, and failed to perform regular megagametogenesis. Q4218 inherited the first two processes of aposporous apomixis from its apomictic 4x parent, but the aposporous embryo sacs that it developed in most of its ovules, did not form seeds. Seed set in the hypotriploid plant was extremely low, even when pollen was supplied abundantly by hand from different diploid and tetraploid genotypes of the *Plicatula* group, including its male and female parents. Moreover, none of the rarely formed seeds germinated in routine germination tests. *In vitro* culture of caryopses hardly improved this performance since only two out of nine germinated. Both seedlings had the chromosome number of the mother plant (2n = 3x -1 = 29), suggesting an apomictic origin in concordance with our observation on megagametogenesis. The question is whether the sterility is due to the high inability of the unreduced egg cell for parthenogenesis and/or because endosperm failed to form.

Many reasons could contribute to the female sterility of this hypotriploid hybrid. However, the failure of the pollen tubes to penetrate and grow into the style supports that pseudogamy could not be completed because endosperm development depends on the fertilization of the polar nuclei. Also, in its mother plant, TK2453 pollen tubes did not reach the micropylar zone of the ovule when pollen from diploid and tetraploid related species were used, and seed set was extremely low under self-, open, and cross-pollination (unpublished data). Therefore, the female infertility in the diploid mother as well as in the hypotriploid could be due to a genetic incompatibility system of the stigma-pollen. Thus, the hypotriploid could inherit the seed sterility from its mother plant. In addition, the parthenogenesis could be nonfunctional, or an interruption of cell or tissue differentiation could occur, but it needs to be studied.

HANNA and DUJARDIN (1982) reported an interspecific triploid hybrid between *Pennisetum americanum* and *P. orientale* that was an obligate apomictic with high expression of apospory (96% of its mature ovules developed aposporous embryo sacs), but with low seed set (1.3%). However, sterility of this nature was not known in *Paspalum* interspecific hybrids until these findings. All of the interspecific triploid hybrids obtained by BURSON (1978, 1979, and 1985), BURSON *et al.* (1973), QUARIN (1983), and QUARIN *et al.* (1984) were sterile, and some only formed a few meiotic sacs.

DARLINGTON (1939) postulated that apomixis was an "escape from sterility" for many highly heterozygous hybrids. Aposporous apomixis was the most likely way through which many *Paspalum* species escaped sterility, as is the case in pentaploid *P. dilatatum* (BURSON 1983), triploid *P. quadrifarium* (QUARIN and LOMBARDO 1986; NORRMANN *et al.* 1989), tetraploid *P. secans* (SNYDER 1957; 1961), and many other apomictic polyploid species with severe meiotic disorders. The hypotriploid analysed showed an irregular meiotic behaviour with more than half of the unpaired chromosomes during the first meiotic division, and with many chromosome losses during the entire process. This irregular behaviour might account for male and female sterility due to severe genetic imbalance of meiotic products. The inheritance of apospory from the male parent might avoid female sterility in the hybrid. However, the apomixis process needs to be completed by all of its components to be an escape from sterility. Evidently, there are factors that could be inherited and could stop the process. Thus, it is not enough to inherit the genetic factor(s) required for expression of the aposporous apomixis because there are other factors that could stop the process and cause infertility. Future studies with other hybrids, which have similar characteristics, would help to improve our understanding of seed sterility in apomictic plants.

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