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Effect of Pollination Timing on the Rate of Apomictic Reproduction Revealed by RAPD Markers in *Paspalum notatum*

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Progeny tests employing molecular markers allow the identification of individuals originated by sexual means among the offspring of a facultative apomict. The objective of this work was to evaluate the effect of the pollination timing on the proportion of sexually formed individuals in progenies of a facultative apomictic *Paspalum notatum* genotype. Progeny families of approx. 30 plants each were generated at five different pollination times: 1–3 d pre-anthesis; at anthesis; and 2, 4 and 6 d post-anthesis. Cytoembryological analyses indicated that approx. 17 % of the ovules carried a meiotic cytologically reduced embryo sac in florets formed simultaneously with those used for crosses. The parental plants and the five F_1 families were analysed using RAPD molecular markers. Ninety-five oligonucleotides were assayed on the progenitors in order to search for male-specific bands. Eight primers presenting clear polymorphic bands were selected for use in the progeny tests. The proportion of sexually produced progeny reached 3·4 % before anthesis and 20 % at anthesis, while pollination after anthesis generated only maternal plants. A second progeny of 97 plants obtained from pollination at anthesis produced 16 off-type plants (16·5 %), of which only one was a B_{III} hybrid ($2n + n$). Our results indicate that pollination at anthesis allows the greatest potential for sexuality to be expressed in this facultative apomictic genotype. When pollination is delayed as soon as 2 d after anthesis, only the aposporous sacs develop endosperm through pseudogamy to set seed.

Key words: Apomixis, *Paspalum notatum*, pollination timing, RAPD fingerprinting.

INTRODUCTION

Paspalum notatum (Bahiagrass) is a perennial rhizomatous forage grass distributed widely in the New World from Central Eastern Mexico to Argentina and throughout the West Indies (Chase, 1929). The species presents several cytotypes that differ in both ploidy level and reproductive behaviour. While the tetraploid races ($2n = 4\ x = 40$) reproduce mostly by obligate apomixis, the diploid form (Pensacola Bahia grass), reproduces sexually and is allogamous due to a self-incompatibility system (Burton, 1946, 1948; Burton and Forbes, 1960). Apomixis in *Paspalum notatum* involves the formation of a non-reduced embryo sac by mitotic division of a nucellar cell (apospory), followed by the fertilization of the central nuclei to form the endosperm (pseudogamy). Even when obligate apomixis is the common form of reproduction in the polyploids, facultative apomixis was occasionally observed in some tetraploid plants of experimental origin (Quarín *et al*., 1984; Burton and Hanna, 1992). Recently, facultative apomixis was also detected in colchicine-induced tetraploid plants (Quarín *et al*., 2002).

Owing to its potential for excellent forage production, *Paspalum* is widely recognized as a promising grass genus (Burton, 1962, 1974). However, the breeding of most *Paspalum* species is still severely restricted by the barrier of apomictic reproduction. Natural populations of apomictic *Paspalum* species are polyploid, fully apomictic and deficient in sexual individuals at the same ploidy level (Norrmann *et al*., 1989; Burson and Hussey, 1998). Nevertheless, the rare facultative apomictic genotypes identified so far are able to generate both maternal and non-maternal offspring and can therefore be used as mother plants in breeding programmes of the species. The development of reliable procedures for the determination of the actual degree of apomictic reproduction in facultative apomictic polyploid races and the unequivocal detection of hybrids are mandatory for the successful completion of such programmes.

Identifying the optimum conditions for hybrid production is also of great relevance to the purpose of improving apomictic cultivars. There has been much speculation about the possibility of controlling the rate of hybrid production through the manipulation of the pollination time (Martínez *et al*., 1994). Precocity in the development of aposporous embryo sacs when compared with meiotic ones has been observed in several facultative apomictic grasses (Savidan, 1991; Leblanc and Savidan, 1994). If the pollination timing is anticipated, this offset may cause a predominance of individuals carrying the maternal genotype in the offspring, simply because the non-reduced embryo sacs are already

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TABLE 1. *Percentage of ovules bearing different types of embryo sacs in* Paspalum notatum *(plant Q4085)*

Embryo sac observed	Percentage of ovules
One meiotic (*Polygonum* type)	10·1
One to several aposporous	79·7
One meiotic + one to several aposporous	6·7
Immature or aborted	3·4

A total of 56 ovules was analysed.

fully mature while the reduced ones are still not receptive to fertilization. Alternatively, it could be hypothesized that normal pollination at anthesis, or some delay in the pollination time, would allow adequate maturation of the meiotic sacs so that they may have at least the same chance as the aposporous ones to develop an embryo.

The objective of this work was to determine whether manipulation of pollination timing could actually modify the expression of apomixis in a facultative apomictic genotype of *Paspalum notatum*. Our approach was based on the generation of five progenies obtained at different pollination times, which were then analysed using molecular markers to assess the genetic origin of the progeny plants: maternal *vs*. off-type. RAPDs had already been successfully used in progeny tests to determine the degree of apomictic reproduction in *Paspalum notatum* (Ortiz *et al*., 1997). In several other apomictic species, molecular markers had been also applied to the assessment of the rate of non-maternal progeny formation (Barcaccia *et al*. 1997; Pessino *et al*., 1999; Arnholdt-Schmitt, 2000). The use of RAPD markers on progeny fingerprints allowed us to detect significant differences in the rate of apomictic reproduction which correlated with the variation of the pollination timing in *Paspalum notatum*.

MATERIALS AND METHODS

Plant material and crosses

Crosses were performed between two tetraploid plants of *P. notatum*: the facultative apomictic accession Q4085 (pistillate parent) was obtained by colchicine treatment of a sexual diploid (Quarín *et al*., 2002), and the obligate apomictic accession Q4117 (pollen donor) introduced from Rio Grande do Sul, Brazil. Five F_1 progeny families of approx. 30 plants each were generated in 1998 at different pollination times: 1–3 d before anthesis (DBA), at anthesis (A) and 2, 4 and 6 d after anthesis (DAA). To corroborate the results at anthesis, the experiment was repeated in 1999. Pre-anthesis pollination was performed using the technique described by Martínez *et al*. (1994). As crosses at anthesis and post-anthesis required emasculation, anthers were removed with the aid of sharp-pointed tweezers in an artificial fog chamber in order to prevent anther dehiscence. Some inflorescences were pollinated immediately and others were bagged to be pollinated 2, 4 and 6 d later with fresh pollen. Emasculated and pollinated inflorescences were maintained in a shaded humid place and bagged to prevent losses due to seed shattering.

RAPD studies

Young leaves from both parents and F_1 progenies were used for the genomic DNA extraction following the protocol described by Ortiz *et al*. (1997). Ninety-five decamers from British Columbia University (Set BC100/3, from 201 to 295) were screened to find polymorphisms between both parents. Eight primers were selected for molecular analyses: primer BC211: 5′-GAAGCGCGAT-3′; primer BC220: 5′-GTCGATGTCG-3′; primer BC229: 5′-CCACCCAGAG-3′; primer BC236: 5′-ATCGTACGTG-3′; primer BC247: 5′-TACCGACGGA-3′; primer BC264: 5′-TCCACCGAGC-3′; primer BC269: 5′-CCAGTTCGCC-3′; primer BC273: 5′-ATTGTCGCCA-3′. RAPDs studies were carried out according the CIMMYT Laboratory Protocol (Hoisington *et al*., 1994) with some modifications. Amplification reactions were performed in a volume of 25 µl containing 1× *Taq* DNA polymerase buffer (Promega), 1·5 mM $MgCl_2$ (Promega), 15 µM of each dNTP, 1·5 U *Taq* DNA polymerase (Promega), 30 ng primer and 20 ng of template DNA. Polymerase chain reactions were carried out using an UNO Biometra thermocycler. The amplification programme was as follows: one cycle at 93 °C for 1 min, followed by 45 cycles of 1 min at 93 °C, 1 min at 36 °C and 1·5 min at 71 °C, and a final cycle at 72 °C for 5 min. Amplification products were separated by electrophoresis in 2 % agarose gels, stained with ethidium bromide, detected by fluorescence on a UV transilluminator and recorded on Polaroid 667 film. Reactions revealing the absence of maternal bands or the presence of paternal bands in the progeny were repeated to check the consistency of the test. The presence or absence of bands was scored visually and included in a matrix as 1 or 0 values, respectively.

Embryological studies

Simultaneously with emasculation and pollination of the mother plant Q4085, inflorescences at anthesis were fixed for 24 h in FAA (18 parts 70 % ethanol : 1 part formaldehyde : 1 part glacial acetic acid) and saved for embryological analysis. Ovaries were dissected, dehydrated, embedded in paraffin wax, sectioned at 12 µm, stained with safranin fast-green series and observed with a light transmission microscope.

Chromosome number of the off-type plants

Plants from the 1999 progeny that were classified as off-type through RAPD fingerprint evaluation were analysed to determine their chromosome number in root-tip cells. Young roots were collected from potted plants, treated in a saturated solution of α-bromonaphthalene for 2 h, hydrolysed in 1 N HCl at 60 °C for 10 min, stained with 1 % basic fuchsin, squashed with a drop of 2 % aceto-orcein on slides and observed by phase contrast microscopy.

TABLE 2. *Off-type plants (sexual origin) detected at different times of pollination in crosses between two tetraploid cytotypes of* Paspalum notatum

Time of pollination	Days	Percentage of caryopses obtained	Number of plants analysed	Number of off-type plants	Off-type plants with	
					$2n = 40$	$2n = 60$
Before anthesis	1–3	30·0	29	1	n.d.	n.d.
At anthesis						
1st progeny (1998)	0	54·2	30	6	n.d.	n.d.
2nd progeny (1999)	0	55·5	97	16	15	1
After anthesis	2	52·9	28	0	–	–
	4	57·0	30	0	–	–
	6	53·0	34	0	–	–

n.d., non-determined.

RESULTS

Reproductive behaviour of the facultative apomictic parent

Inflorescences of plant Q4085 showed different types of ovules as expected for a facultative apomictic plant (Table 1). All the inflorescences analysed in this plant were formed simultaneously with those used for the crosses. Aposporous embryo sacs were identified easily due to the lack of antipodal cells and because they showed varying positions and orientation within the ovule. We found that approx. 80 % of the ovules produced one or usually several aposporous embryo sacs. On the other hand, approx. 10 % of ovules showed one meiotic embryo sac typical of sexual reproducing grasses. Some ovules (6·7 %) showed one meiotic sac together with one to several aposporous sacs and, only a few (3·4 %), immature underdeveloped embryo sacs or an absence of embryo sacs. When considering ovaries producing only meiotic sacs together with those containing mixed meiotic and aposporous sacs, the potential for sexual reproduction varied from 10 % to approx. 17 % and for apomictic reproduction from approx. 80 % to more than 86 %. The reproductive behaviour of Q4117 plant had already been determined in a previous study (Ortiz *et al*., 1997).

Genesis of progeny families

Non-maternal plants (originated sexually) could be generated: (1) by fertilization of a reduced egg cell by a sperm nucleus of the male parent forming a B_{II} hybrid ($n + n$); (2) by fertilization of an unreduced egg cell of aposporous embryo sacs to form a B_{III} hybrid ($2n + n$); or (3) by occasional self-fertilization of an egg cell of a meiotic embryo sac. In each case, reproduction would be sexual and thus the off-type plants or the so-called aberrant in the progeny indicated sexual reproduction. Dihaploids were not expected to be formed, since haploid parthenogenesis has never been reported in this species. Moreover, several attempts to produced dihaploids from apomictic tetraploid *P. notatum* crosses using different protocols failed in our laboratory (unpubl. res.). In addition, chromosome number determination eliminated the possibility of overlooking haploid parthenogenesis (see below).

RAPD analysis

RAPD analyses were used to analyse the progeny generated. Ninety-five decamers were assayed to identify polymorphisms between the parents. Out of the total number of oligonucleotides analysed, only seven did not produce amplification products. Among the remaining 88, 25 showed polymorphisms between both parents. Eight primers that yielded clear and reproducible RAPD bands in both parents were selected to perform the molecular studies (see Materials and Methods). The selected primers generated seven bands specific to the male parent and five to the female one. Additional non-polymorphic bands displayed by both parental genotypes were also considered, since the lack of such bands in the progeny may also indicate the occurrence of recombination. Hybrids (B_{II} and B_{III}) were detected by the presence of male specific bands and eventually confirmed by the lack of maternal bands. A seedling was considered to be of sexual origin when its fingerprints lacked at least one band of those present in the maternal plant Q4085 (hybrids or eventually self-pollinated escapes), or when it showed a band specific to the pollen donor plant Q4117 (hybrids).

Initially, all 151 individuals belonging to the five F_1 progenies obtained at different pollination times in 1998 were analysed by RAPD markers (Table 2). Only one non-maternal plant (3·4 %) was identified by BC211 and BC229 primers in a progeny of 29 plants obtained from pollination 1–3 d before anthesis. Since both BC211 and BC229 detected male-specific bands in the F_1 individual, it was classified as a hybrid. Pollination performed at anthesis generated a progeny family consisting of 20 % of non-maternal plants: six out of 30 seedlings analysed were of sexual origin, while the remaining 24 plants always showed the maternal fingerprints (Fig. 1). Three individuals of sexual origin (H4, H18 and H29) were identified using primer BC220; these lacked a band present in the female progenitor (Fig. 1A). Three additional non-maternal plants (H8, H17 and H20) were differentiated using primer BC269 (Fig. 1B) by detecting a band specific to the male parent. Amplification with primer BC236 confirmed the hybrid origin of plants H8 and H20 (Fig. 1C). Oligonucleotides BC229, BC264 and BC273 always spotted those hybrids

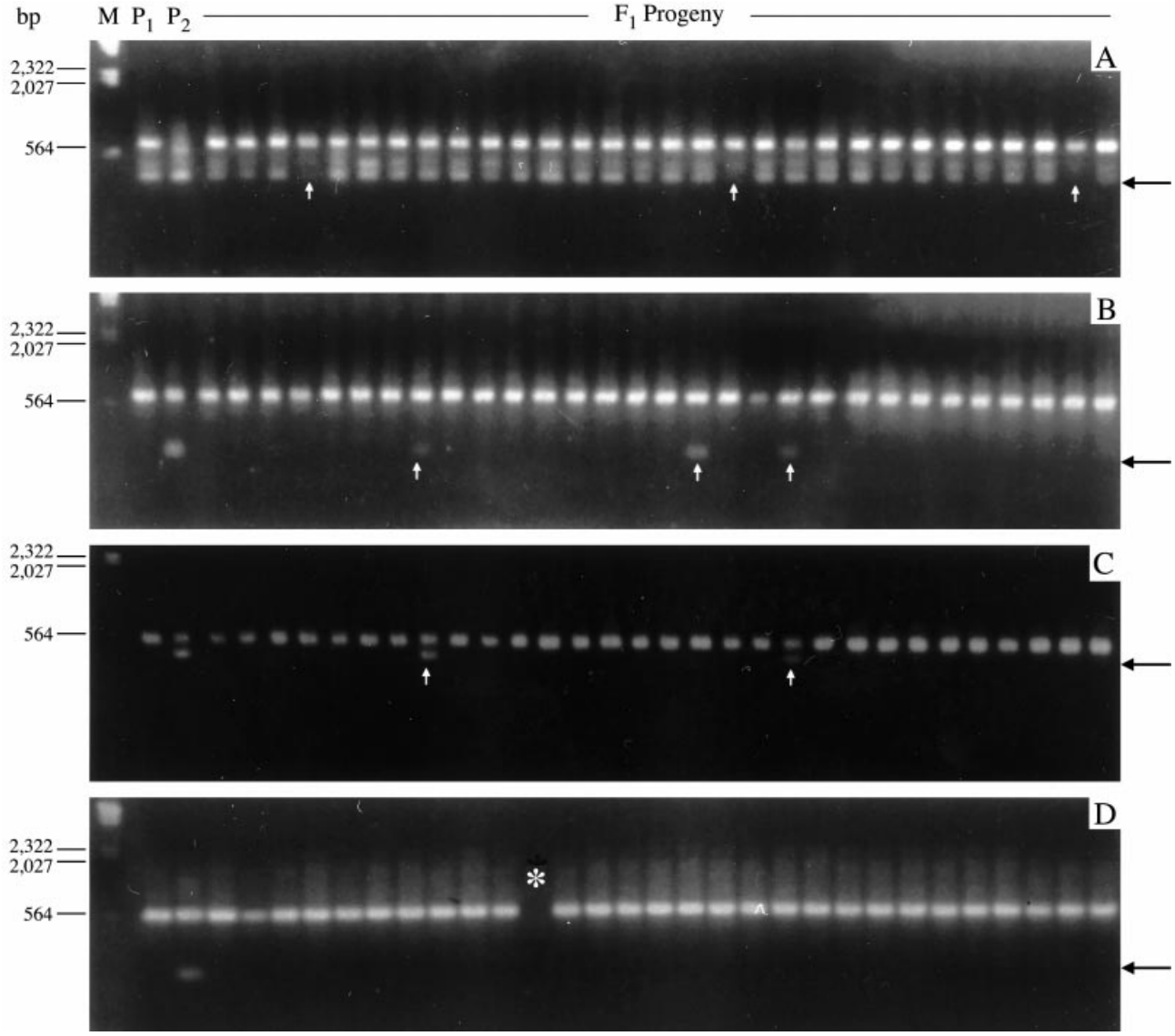

FIG. 1. Genetic fingerprints of both parents and their progeny family obtained by pollination at anthesis (A, B and C) and 6 d after anthesis (D). P_1 female parent. P_2 male parent. A, Amplifications obtained using primer BC220, which detected three off-types plants (sexual origin) by the absence of a maternal band; B, RAPD pattern obtained with primer BC269, where three other non-maternal individuals were spotted by amplification of a band originated in the male parent; C, two hybrids detected with primer BC236 that had already been marked by BC269; D, no off-types plants were detected using the primer BC269 (the same primer as used in B). M indicates the lane where the molecular marker (lambda DNA digested with *Hin*dIII) was loaded. Black arrows indicate the amplification fragments used to discriminate between maternal (apomictic origin) and non-maternal plants (sexual origin). White arrows mark the individuals of sexual origin detected by the corresponding primer. The asterisk indicates a lane where no amplification was observed.

that had been detected by BC269, while BC211 and BC247 failed to identify non-maternals. Exclusively maternal fingerprinting patterns were observed in progenies originated from crosses performed at 2, 4 and 6 d after anthesis (Fig. 1D) with all the primers used.

The selected primers were assayed in all the progenies obtained to search for non-maternals or apomictic offsprings. Considering all the possible genotypic constitution of the loci detected (+−−−, ++−−, +++−, ++++), the probability of a hybrid being detected by amplification of a male-specific band would be $P > 0{\cdot}5$ for each marker used. Thus, the probability of detecting each hybrid with at least one of the seven male-specific markers used would be $P > 0{\cdot}992$. Additional maternal and non-polymorphic bands observed to be absent in the progeny further lowered the possibility of missing a non-maternal genotype.

Contingency tests verified the statistical significance of differences among the number of off-type plants recovered. Differences observed between the number of aberrant before and at anthesis was significant ($\chi^2 = 3{\cdot}87$; $P < 0{\cdot}05$). Variations in number of non-maternals observed among progenies arising from pollination after anthesis and at

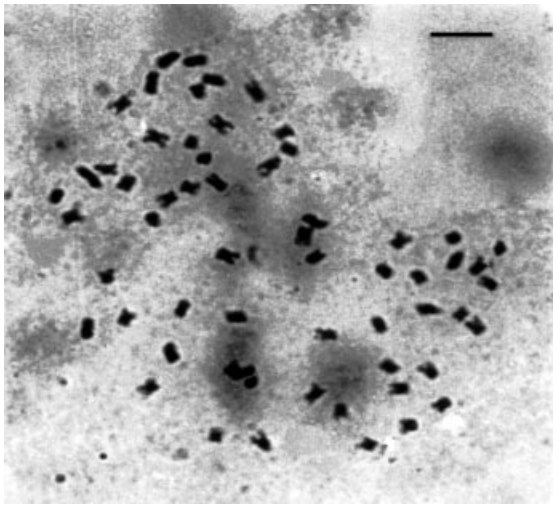

FIG. 2. Sixty chromosomes in mitotic metaphase of the B_{III} hybrid ($2n + n$) of *Paspalum notatum*. Bar = 10 μm.

anthesis were significant ($\chi^2 = 6{\cdot}24$, $P < 0{\cdot}025$) for the progeny obtained at 2 d after anthesis or highly significant ($\chi^2 = 6{\cdot}66$, $P < 0{\cdot}01$; $\chi^2 = 7{\cdot}5$, $P < 0{\cdot}01$) for progenies obtained at 4 and 6 d after anthesis, respectively. In all cases, the d.f. considered was 1.

A second larger progeny was developed in 1999 by pollination at anthesis to confirm the observed rate of apomictic reproduction and assess the ploidy level of the non-maternal plants (Table 2). Ninety-seven plants were analysed by RAPD studies with the same primers as in 1998. Using primer BC220, 12 individuals of sexual origin were identified, that lacked a band present in the female parent. Decamer BC264 detected three additional non-maternal plants and corroborated one already identified by BC220, which represented a band specific to the male parent. Two off-type plants were detected by primer BC211 (one of them already identified by primer BC264). Oligonucleotides BC236, BC269 and BC273 spotted non-maternal plants previously revealed by the primers specified above. No off-type plants were detected using primers BC229 and BC247. The proportion of aberrant individuals (16·5 %) observed in the 1999 progeny was comparable with that scored in the 1998 progeny (20 %) ($\chi^2 = 0{\cdot}19$; $P < 0{\cdot}30$).

Chromosome number

Chromosome counting was carried out on the 16 aberrant plants of the 1999 progeny. One of them proved to be a B_{III} hybrid ($2n + n$) with $2n = 60$ chromosomes (Table 2, Fig. 2). The remaining off-types had $2n = 40$ chromosomes, certainly due to the syngamy of two reduced gametes ($n + n$). These results confirm the non-appearance of dihaploids in the F_1 progeny.

DISCUSSION

The tetraploid female parent was produced by colchicine treatment of a diploid. This diploid must have been heterozygous (+ –) for some RAPD markers, so that the female parent would be + + – – and therefore able to produce – – gametes and some descendants lacking the marker due to sexual reproduction. Similarly, the male parent must be heterozygous for all the observed markers as none of the primers detected 100 % of the non-maternal plant.

RAPD fingerprinting is an efficient screening tool for the identification of off-type progeny and the assessment of the mode of reproduction in apomictic species, mainly because of its reliable discriminatory capacity among maternal and non-maternal plants in progeny tests. In addition, the use of molecular markers allows the actual proportion of sexuality in a given population to be assessed. The potential for sexual reproduction determined by cytoembryology and the real proportion of plants with sexual origin showed a good correlation with a previous study on a facultative apomictic plant of *P. notatum* (Ortiz *et al*., 1997). However, our results show that important deviations may occur from the potential degree of sexuality as assessed by embryological techniques and the actual proportion of individuals generated by sexuality. Before considering the effect of timing of pollination, there are intrinsic factors that may cause changes in the degree of effective sexual reproduction. For example, the average number of spikelets that form caryopses in tetraploid races of *P. notatum* is usually low varying from 40 to 70 % (Burton, 1946), meaning that a substantial proportion of ovaries fail to develop into a seed. This failure may be due to destructive competitiveness of multiple aposporous embryo sacs in a single ovule or it may result from lack of fertilization of meiotic sacs.

Our results indicate that pollination timing has a clear influence on the rate of apomictic reproduction. When pollination was performed 1–3 DBA, only 30 % of spikelets formed caryopses and most of the plants recovered originated from aposporous sacs. The low seed set may be due to mechanical damage during the pollination procedures or to the presence of some still immature embryo sacs, especially those of meiotic origin. This evidence agrees with the previous observation that the aposporous sacs reach maturity more precociously than meiotic sacs in facultative apomictic grasses (Leblanc and Savidan, 1994). Thus, early pollination favours apomictic reproduction in *P. notatum*, and probably the only hybrid plant that we obtained from 1–3 DBA pollination could have been a $2n + n$ plant or an $n + n$ hybrid formed approx. 1 DBA. Unfortunately, we failed to count the chromosome of this hybrid plant before it died.

Emasculation and cross-pollination at anthesis balanced the opportunities for sexual and apomictic reproduction to occur. The percentage of off-type individuals in the progeny was correlated with the full potential for sexual reproduction as assessed through embryological analysis. Counting of chromosomes revealed that fertilization of a non-reduced egg cell of an aposporous embryo sac was a rare event in comparison with the 46 % of $2n + n$ hybrids reported to be obtained when an obligate apomictic plant was pollinated 3 DBA (Martínez *et al*., 1994). At anthesis, the meiotic embryo sacs have probably attained an appropriate stage of development for syngamy to occur, while fertilization of the

non-reduced egg-cell of aposporous embryo sacs is already severely restricted.

Remarkably, the egg cell (reduced or non-reduced) and sperm cell syngamy was restricted absolutely from 2 d after anthesis. However, second fertilization events concerning polar nuclei and the remaining sperm nucleus of the pollen tube surely occurred. This allowed further development of aposporous embryo sacs through pseudogamy to generate progeny only by asexual means (Burton, 1948; Quarín, 1999). The restriction of syngamy after anthesis would most probably concern only those genotypes carrying the genes for apomixis, since we have previously observed that sexual tetraploid plants of *P. notatum* produced seed when emasculated and pollinated several days after anthesis (unpubl. res.). According to these results, we suggest that the same mechanism which prevents the fertilization of unreduced egg cells in mature aposporous embryo sacs at anthesis could also be occurring in meiotic sacs of facultative apomictic genotypes at a latter developmental stage. Additional experiments may allow us to corroborate whether the factors that operate preventing fertilization of the 2*n* egg cell at anthesis in apomictic genotypes could also affect the egg cell of their occasional meiotic embryo sacs.

Our results indicate that it is possible to manipulate the timing of pollination in facultative apomictic plants to obtain progenies with variable representation of maternal genotypes. If the aim is the acquisition of new genotypes, pollination should always be performed at anthesis to increase the chances of producing non-maternal offspring. This procedure can be assayed to obtain a maximal expression of sexuality in other facultative apomictic *Paspalum* species with excellent forage potential, for example those in the Plicatula and Dilatata groups. No sexual genotypes at the same ploidy level of the apomictic species have been found for these groups in nature. Thus, even a modest potential for sexual reproduction could be ascertained through embryological studies using the clearing technique. Emasculation and crossing would produce some new apomictic genotypes for agronomic evaluation and selection. The identification of non-maternal plants can be greatly enhanced by the use of molecular markers following an experimental design similar to the one used in this work.

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