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Analysis of variation for apomictic reproduction in diploid *Paspalum rufum*

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• *Background and Aims* The diploid cytotype of *Paspalum rufum* (Poaceae) reproduces sexually and is self-sterile; however, recurrent autopolyploidization through $2n + n$ fertilization and the ability for reproduction via apomixis have been documented in one genotype of the species. The objectives of this work were to analyse the variation in the functionality of apomixis components in diploid genotypes of *P. rufum* and to identify individuals with contrasting reproductive behaviours.
• *Methods* Samples of five individuals from each of three natural populations of *P. rufum* (designated R2, R5 and R6) were used. Seeds were obtained after open pollination, selfing, conspecific interploidy crosses and interspecific interploidy self-pollination induction. The reproductive behaviour of each plant was determined by using the flow cytometric seed screen (FCSS) method. Embryo sacs were cleared using a series of ethanol and methyl salicylate solutions and observed microscopically.
• *Key Results* In open pollination, all genotypes formed seeds by sexual means and no evidence of apomeiotic reproduction was detected. However, in conspecific interploidy crosses and interspecific interploidy self-pollination induction, variations in the reproductive pathways were observed. While all plants from populations R2 and R6 formed seeds exclusively by sexual means, three genotypes from the R5 population developed seeds from both meiotic and aposporous embryo sacs, and one of them (R5#49) through the complete apomictic pathway (apospory + parthenogenesis + pseudogamy). Cytoembryological observations revealed the presence of both meiotic and aposporous embryo sacs in all the genotypes analysed, suggesting that parthenogenesis could be uncoupled from apospory in some genotypes.
• *Conclusions* The results presented demonstrate the existence of variation in the functionality of apomixis components in natural diploid genotypes of *P. rufum* and have identified individuals with contrasting reproductive behaviours. Genotypes identified here can be crossed to generate segregating populations in order to study apomixis determinants at the diploid level. Moreover, analysis of their expression patterns, quantification of their transcript levels and an understanding of their regulation mechanisms could help to design new strategies for recreating apomixis in a diploid genome environment.

Key words: Apomixis, diploid genotype, *Paspalum rufum*, Poaceae, reproduction, seed development, plant mating systems.

INTRODUCTION

Paspalum is a large genus of the grass family (Poaceae) that includes about 350 species, many of which are major constituents of natural pastures of tropical and sub-tropical regions of the Americas (Zuloaga and Morrone, 2005). Several species of the genus form multiploid complexes where diploid cytotypes reproduce sexually and are self-sterile, while polyploids (mainly tetraploids) are pseudogamous self-fertile aposporous apomicts (Quarin, 1992). *Paspalum rufum* Nees ex Steud. is a robust erect perennial grass native to Paraguay, southern Brazil, Uruguay and north-eastern Argentina (Quarin *et al.*, 1998). The diploid race ($2n = 2x = 20$) shows regular meiosis, reproduces mainly sexually and is self-sterile. The tetraploid cytotype ($2n = 4x = 40$) has irregular meiotic behaviour, with chromosomes associating mainly as bivalents and quadrivalents, and reproduces by pseudogamous aposporous apomixis

(Norrmann *et al.*, 1989). There is evidence indicating that tetraploids have an autoploid origin (Quarin *et al.*, 1998).

Apomixis is an asexual reproduction through seeds that generates clonal progeny (Nogler, 1984). Gametophytic apomixis comprises three main components: unreduced embryo sac formation (apomeiosis) from the megaspore mother cell itself after a failure in meiosis (diplospory) or from a nucellar cell (apospory); fertilization-independent embryogenesis from the unreduced egg cell (parthenogenesis); and autonomous endosperm formation or after polar nuclei fertilization (pseudogamy). Although there is a strong link between gametophytic apomixis and polyploidy (Savidan, 2000), several species of the genus *Paspalum* showed ovules bearing aposporous embryo sacs along with the typical meiotic ones at the diploid level. The presence of aposporous sacs suggested some potential for apomictic reproduction of diploid cytotypes (Quarin, 1986; Quarin and Norrmann, 1987; Norrmann *et al.*, 1989; Quarin *et al.*, 2001).

Embryological observation of the genotype Q3754 of *P. rufum* revealed that between 8·8 and 26·8 % of their ovaries included one meiotic plus one aposporous embryo sac (Norrmann *et al.*, 1989). Also, this diploid genotype formed tetraploid B$_{III}$ hybrids in crosses with a tetraploid pollen donor, indicating that non-reduced embryo sacs were able to form seeds in conspecific interploidy crosses (Norrmann *et al.*, 1994). Genetic analysis carried out with the aid of molecular markers showed that after induced self-pollination (using mentor pollen from 4*x* *Paspalum urvillei*), Q3754 generated up to 5 % of its progeny by apomixis (Siena *et al.*, 2008). Functional apomeiotic diplosporous embryo sacs at the diploid level were also found in species of the genus *Boechera* (Schranz *et al.*, 2005; Aliyu *et al.*, 2010).

The functionality of apomeiotic embryo sacs and the complete route for apomixis reproduction at the diploid level have both ecological and practical importance. From the ecological point of view, they can explain the origin of new polyploids (triploids and tetraploids) within a diploid population and how variability is transmitted from diploids to higher ploidy levels (Quarin *et al.*, 1989; Siena *et al.*, 2008). From a practical standpoint, understanding the fundamental aspects of apomixis determinant(s) in diploids could facilitate the isolation of the gene(s) responsible for the trait, avoiding the difficulties associated with polyploid environments (where the character is normally expressed), and the design of strategies for the transfer of the character to diploid crops. In this context, the evaluation of variability in the functionality of components of apomixis in natural diploid genotypes of *P. rufum* is worthy of interest.

Facultative apomictic plants may originate several different types of progeny by different reproductive pathways (Nogler, 1984; Savidan, 2000). The operational seed formation routes in these types of individuals can be inferred and quantified using the flow cytometric seed screen (FCSS) method, by which the relative DNA contents of the embryo and endosperm are measured (Matzk *et al.*, 2000; Bicknell *et al.*, 2003). Because both meiotic and aposporous embryo sacs of most *Paspalum* species contain two polar nuclei, the FCSS technique can be used in individual or bulked seeds to determine the different reproductive pathways of individual plants. This methodology has been applied to determine the mode of reproduction in several species of the genus (Siena *et al.*, 2008; Sartor *et al.*, 2009; Aguilera *et al.*, 2011; Rebozzio *et al.*, 2011; Sartor *et al.*, 2011; Hojsgaard *et al.*, 2013).

The objectives of this work were to analyse the variation in the functionality of aposporous embryo sacs and the capacity for apomictic reproduction in diploid genotypes of *P. rufum* and identify individuals with contrasting reproductive behaviour useful for genetic and expression analysis of the trait at the diploid level.

MATERIALS AND METHODS

Plant material and seed set analysis

This study was based on three natural diploid ($2n = 2x = 20$) populations of *Paspalum rufum*, named R2, R5 and R6, previously studied by Sartor *et al.* (2011). Thirty plants from each population were cultivated in a field nursery at the National University of Northeast, Corrientes, Argentina. Five plants from each population chosen at random were employed. Seed in each plant were recovered after: (1) open pollination; (2) self-pollination; (3) conspecific interploidy crosses with pollen of a tetraploid cytotype ($2n = 4x = 40$); and (4) interspecific self-pollination induction by adding mentor pollen from tetraploid ($2n = 4x = 40$) *Paspalum urvillei*. For crosses and induction of self-pollination, inflorescences at anthesis were dusted with the corresponding pollen source early in the morning just after anthesis, and then covered with glassine bags to prevent contamination. Self-pollinations were carried out by bagging young inflorescences before anthesis and keeping them covered until maturity. Seed set in each treatment was determined as the percentage of filled spikelets over the total spikelets scored. Comparisons of the average percentage of filled spikelets between population samples were performed using the non-parametric Kruskal–Wallis test of the statistical package Infostat (Di Rienzo *et al.*, 2008); *P*-values <0·05 were considered significant.

Reconstruction of seed formation pathways by the FCSS method

The reproductive pathways of seed formation were determined using the FCSS method (Matzk *et al.*, 2000). Briefly, seed bulks from open pollination (five seeds per bulk) or individual seeds from conspecific interploidy and interspecific interploidy crosses were chopped in 0·5 mL of extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer, Partec, Münster, Germany). The mixture was filtered through a 30 μm nylon mesh, transferred to sample tubes and incubated for 1 min before adding 1·5 mL of 4′,6-diamidino-2-phenylindole (DAPI; CyStain UV Precise P Staining Buffer, Partec). The DNA content of nuclei (C value) was determined by measuring the fluorescence intensity of DAPI-stained nuclei using a Partec PA II flow cytometer (Partec GmbH), with the detector operating at 355 nm. About 3000 nuclei were measured per sample. Single seed from a diploid sexual plant of *P. rufum* (a plant for which the chromosome number was established by chromosome counts in root tips) was used as a reference to correct for peak shifts during the examination. Data analysis was performed using the PA II's Partec FloMax software. Average values of fluorometric measurements were compared using a one-way analysis of variance (ANOVA) test of the statistical package Infostat (Di Rienzo *et al.*, 2008); *P*-values <0·05 were considered significant. The reproductive pathways were reconstructed by determining the ploidy levels of the embryo and the endosperm of each sample (Sartor *et al.*, 2011). The DNA peak index was estimated as the DNA content of the embryo cells in relation to that of the endosperm cells (Matzk *et al.*, 2000).

Embryological analysis

Inflorescences at anthesis were collected and fixed in FAA (70 % ethanol, glacial acetic acid, formaldehyde, 90:5:5) and transferred to 70 % ethanol for at least 24 h. Dissected pistils were passed through a series of ethanol and methyl salicylate solutions following the protocol described by Young *et al.* (1979). Cleared ovules in methyl salicylate were observed with a light transmission Leica DIASTAR microscope equipped with a differential interference contrast (DIC) system and a digital camera (Leica Microsystems, Wetzlar, Germany).

Embryo sacs showing one egg cell, two synergids, two polar nuclei and a group of antipodal cells at the chalazal pole were considered to be of the meiotic type, i.e. derived from meiotic division of a megaspore mother cell. Embryo sacs showing an egg cell, two synergids and two polar nuclei but lacking antipodal cells were considered to be the aposporous type, i.e. derived from mitotic division of nucellar cells called aposporous initials.

RESULTS

Seed set and seed development pathway analysis

As expected, all plants produced the highest seed set in open pollination, although variation was detected between genotypes within the three groups of plants. On average, seed set of plants from populations R2, R5 and R6 was 66·93 % ($\pm$20·95), 45·72 % ($\pm$20·49) and 57·48 % ($\pm$24·07), respectively (Table 1). Comparison of the average percentage seed formation between the three groups of plants showed no significant differences (at $P < 0.05$). Under self-pollination, very low percentages of filled caryopses were obtained. The seed set of individuals from populations R2, R5 and R6 were on average 0·35 % ($\pm$0·50), 0·57 % ($\pm$0·35) and 1·25 % ($\pm$1·59), respectively, without significant differences between populations samples (at $P < 0.05$) (Table 1). These results confirmed that diploids are mainly outbreeders due to the presence of a self-incompatibility system, although it is not fully effective, as reported by Norrmann *et al.* (1994). Conspecific crosses with $4x$ individuals showed in most cases proportions of filled caryopses higher than the values obtained in selfing. On average, seed set of samples of populations R2, R5 and R6 were 0·82 % ($\pm$0·36), 1·20 % ($\pm$1·11) and 2·17 % ($\pm$2·28), respectively, with no significant differences (at $P < 0.05$) between groups (Table 1). On the other hand, only six individuals could be tested in interspecific interploidy-induced self-pollination.

TABLE 1. *Seed set of diploid* P. rufum *genotypes under different pollination conditions*

Population	Individual*	Seed set (%)[†]		
		Open pollination	Self-pollination	Crossed with $4x$ *P. rufum*
R2	#5	92·40	0·00	0·91
	#8	78·95	0·00	0·90
	#15	51·00	0·45	0·73
	#42	71·59	1·18	1·27
	#51	40·72	0·14	0·28
R5	#13	60·30	0·16	0·23
	#22	60·80	1·02	2·56
	#38	19·50	0·69	0·10
	#49	60·50	0·26	2·15
	#51	27·50	0·71	0·95
R6	#13	40·67	0·07	0·33
	#18	24·71	0·25	1·00
	#45	71·88	3·85	4·86
	#53	83·29	0·39	4·45
	#61	66·86	1·68	0·25

*According to Sartor *et al.* (2011).
[†]Percentage of spikelets filled with caryopses.

Plants R2#5 and R6#53 showed seed sets similar to selfing (0·27 and 0·09 %, respectively), but plants R5#22, R5#49, R5#51 and R6#45 showed higher percentages, ranging from 5·43 to 33·64 %, reflecting a different effect of the mentor pollen in the genotypes tested.

The seed formation pathways operating under the different pollination conditions were determined by the FCSS method (Matzk *et al.*, 2000). The possible DNA embryo:endosperm ratios of seeds, considering the fertilization of both a meiotic and aposporous embryo sac with reduced pollen from a diploid or a tetraploid donor, is depicted in Fig. 1. Usually, apomictic seed formation in most *Paspalum* species implies single fertilization, i.e. parthenogenetic development of the unreduced egg cell and pseudogamy (fertilization of the unreduced polar nuclei); however, double fertilization may eventually occur in aposporous sacs with the subsequent increment of the ploidy level in the resulting seed.

Histograms of all seeds recovered from open pollination (at least 50 seeds per plant) showed exclusively 2:3 peaks corresponding to normal sexual reproduction (i.e. the double fertilization of a meiotic embryo sac with reduced pollen from a diploid donor) (Fig. 2A), and no differences were found between genotypes. However, histograms of the caryopses generated in conspecific interploidy- and interspecific interploidy-induced self-pollination varied between population samples and genotypes (Table 2). Under these conditions, plants from populations R2 and R6 formed seeds that showed histograms exclusively with a 2:3 DNA embryo:endosperm ratio (Fig. 2A, Table 2). Thus, although pollen from $4x$ *P. rufum* (or $4x$ *P. urvillei*) was present, all seeds derived from the fertilization of a meiotic embryo sac by a reduced self-gamete (S_{II}) (Table 2). In contrast, three genotypes of population R5 generated seeds with histograms showing 2:3; 3:5 and 4:6 DNA embryo:endosperm ratios (Table 2; Fig. 2A, B), and one of them (R5#49) also produced histograms with 3:4 and 2:6 ratios, the latter after pollination with tetraploid *P. urvillei* (Table 2, Fig. 2E). Histograms with a 3:4 DNA ratio must be derived from the double fertilization of a meiotic embryo sac by reduced pollen from the tetraploid donor (triploid B_{II} embryo). Histograms with 3:5 and 4:6 DNA ratios can be explained by seeds derived from the fertilization of functional aposporous embryo sacs by reduced male gametes from a diploid (forming a triploid S_{III} embryo) or a tetraploid pollen donor (tetraploid B_{III} embryo), respectively. A DNA embryo:endosperm ratio of 2:6 can only be explained by the parthenogenetic development of a non-reduced egg cell (M embryo) of an aposporous embryo sac and the fertilization of the non-reduced polar nuclei by reduced pollen from the tetraploid *P. urvillei*. Quantitative values of fluorometric determinations and peak indexes for each type of progeny are shown in Supplementary Data Tables S1, S2 and Fig. S1.

Analysis of the functionality of the aposporous embryo sacs and the capacity for apomixis showed differences between genotypes of population R5. While plants R5#22 and R5#38 formed seeds only from the fertilization of a meiotic embryo sac with reduced self-pollen, plants R5#13, and R5#51 produced about 12 % of their seeds from their aposporous embryo sacs (Fig. 3A). In both plants, one seed corresponded to an S_{III} individual and the other to a B_{III} hybrid (Table 2). Furthermore, plant R5#49 generated 35·15 % of its seeds from aposporous embryo sacs (Fig. 3A), 36 % of which corresponded to S_{III} and 63 % to B_{III}
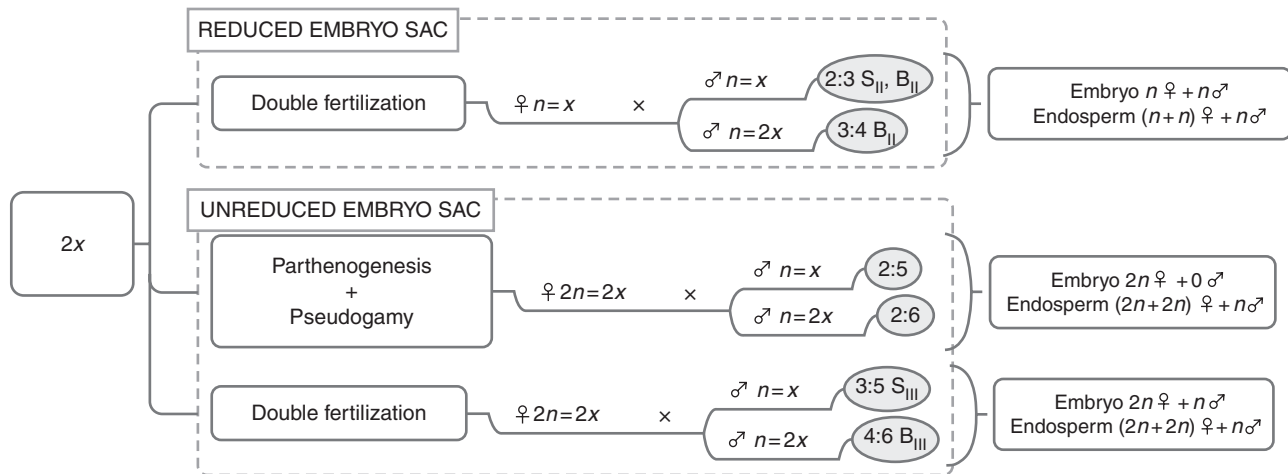
Fig. 1. Theoretical developmental pathways of seed formation in diploid ($2n = 2x = 20$) *Paspalum rufum* carrying functional meiotic and aposporous embryo sacs, fertilized with diploid and tetraploid pollen donors. A meiotic embryo sac fertilized by reduced pollen from a diploid (by crossing or selfing) will generate seeds with a DNA embryo:endosperm ratio = 2:3 (B_{II} or S_{II}, respectively). A meiotic embryo sac fertilized by a reduced pollen from a tetraploid could give rise to a triploid B_{II} hybrid with a DNA embryo:endosperm ratio = 3:4. On the other hand, a functional aposporous embryo sac may develop a diploid embryo by parthenogenesis ($2n + 0$) and the endosperm by pseudogamy, generating maternal progeny. In this case, polar nuclei are fertilized with a reduced gamete from a diploid or a tetraploid, generating seeds with DNA embryo:endosperm ratios of 2:5 or 2:6, respectively. Moreover, functional aposporous embryo sacs can undergo double fertilization with pollen from a diploid or a tetraploid plant, generating seeds with a DNA embryo:endosperm ratio of 3:5 (B_{III} or S_{III}) or 4:6 (B_{III}), respectively. x, ploidy level or basic number of chromosomes; n, gametic chromosome number; B_{II}, diploid hybrid derived from sexual reproduction; B_{III}, hybrid derived from the fertilization of an unreduced egg cell by a haploid gamete; S_{II}, diploid individual derived from selfing; S_{III}, triploid individual derived from the fertilization of an unreduced egg cell by selfing. The different progeny classes were named according to Bicknell *et al.* (2003).

progeny types (Table 2; Fig. 3). The examination of seeds derived from crosses with $4x$ *P. urvillei* showed that plant R5#49 generated 15 % of its seeds by apomixis (M progeny; Fig. 3B, Table 2), while plants R5#22, R5#51, R6#45 and R6#53 formed all of their caryopses by selfing (S_{II}; Table 2, Fig. 3B). The recovery of maternal progeny after interspecific interploidy-induced self-pollination in the species was reported previously by Siena *et al.* (2008).

Cytoembryological analysis

Cytoembryological analyses of ovules at anthesis were carried out in plants from the three population samples. At least 50 ovules per plant were analysed for the presence of a single meiotic embryo sac, a single or multiple aposporous embryo sacs, or one meiotic sac plus an aposporous sac. In this survey, plants that had showed contrasting reproductive behaviours (exclusively by sexuality or by sexuality and apomixis) in the FCSS analysis were selected. According to this criterion, two plants from population R2 (R2#5 and R2#51) and three plants from population R6 (R6#18, R6#45 and R6#53) were selected because they generated all their seeds sexually, suggesting no potential for apomeiotic seed development (Table 2). On the other hand, plant R5#49 was selected because several of their seeds derived from the double fertilization of functional aposporous embryo sacs and by the apomictic pathway (Table 2). Cytological observations of genotype R6#45 showed that out of 104 mature ovules analysed, 90·38 % had a single meiotic sac, 3·85 % were aborted ovules with no sac at all, but unexpectedly, 5·77 % (six ovules) showed one meiotic sac plus an aposporous embryo sac (Table 3). Similar results were found in the other plants from populations R2 and R6. Ovaries bearing both meiotic plus one aposporous embryo sac were detected in all

genotypes, ranging from 1·81 to 12·96 %, indicating that these genotypes have the capability for apospory. Furthermore, R6#53 also has 2·35 % of ovaries carrying single aposporous embryo sacs (Table 3). Analysis of plant R5#49 showed as expected both types of embryo sacs. From a total of 123 ovules observed, 58·53 % bore exclusively a meiotic embryo sac, 28·45 % were aborted, 12·19 % showed one meiotic sac together with an aposporous sac and 0·81 % (only one ovule) showed a single aposporous embryo sac (Fig. 4A, B).

DISCUSSION

In this work, we performed an experimental-based analysis to determine the existence of variability in the functionality of gametophytic apomixis components in diploid genotypes of *Paspalum rufum* and to identify individuals with contrasting reproductive behaviours. Plants displaying differences in the capacity for apomixis can be used for genetic analysis of the trait at the diploid level. Different pollination conditions were applied in order to stimulate all the potential pathways of seed formation reported in the diploid cytotype of the species (Norrmann *et al.*, 1994; Siena *et al.*, 2008). Seed set analyses confirmed that diploids are mainly outbreeders because of the presence of a self-incompatibility system, and corroborated the mentor effect caused by pollen from conspecific tetraploid cytotypes. Accordingly, the presence of pollen of higher ploidy allowed a partial breakage of the self-incompatibility system and the recovery of seeds from selfing. In some cases, the fertilization also occurs by a reduced gamete from the tetraploid donor. These results are in agreement with previous reports in other *Paspalum* species, such as *P. equitans*, *P. ionanthum* and *P. notatum*, in which the addition of foreign pollen increased the number of self progeny (Quarin and Norrmann, 1987;
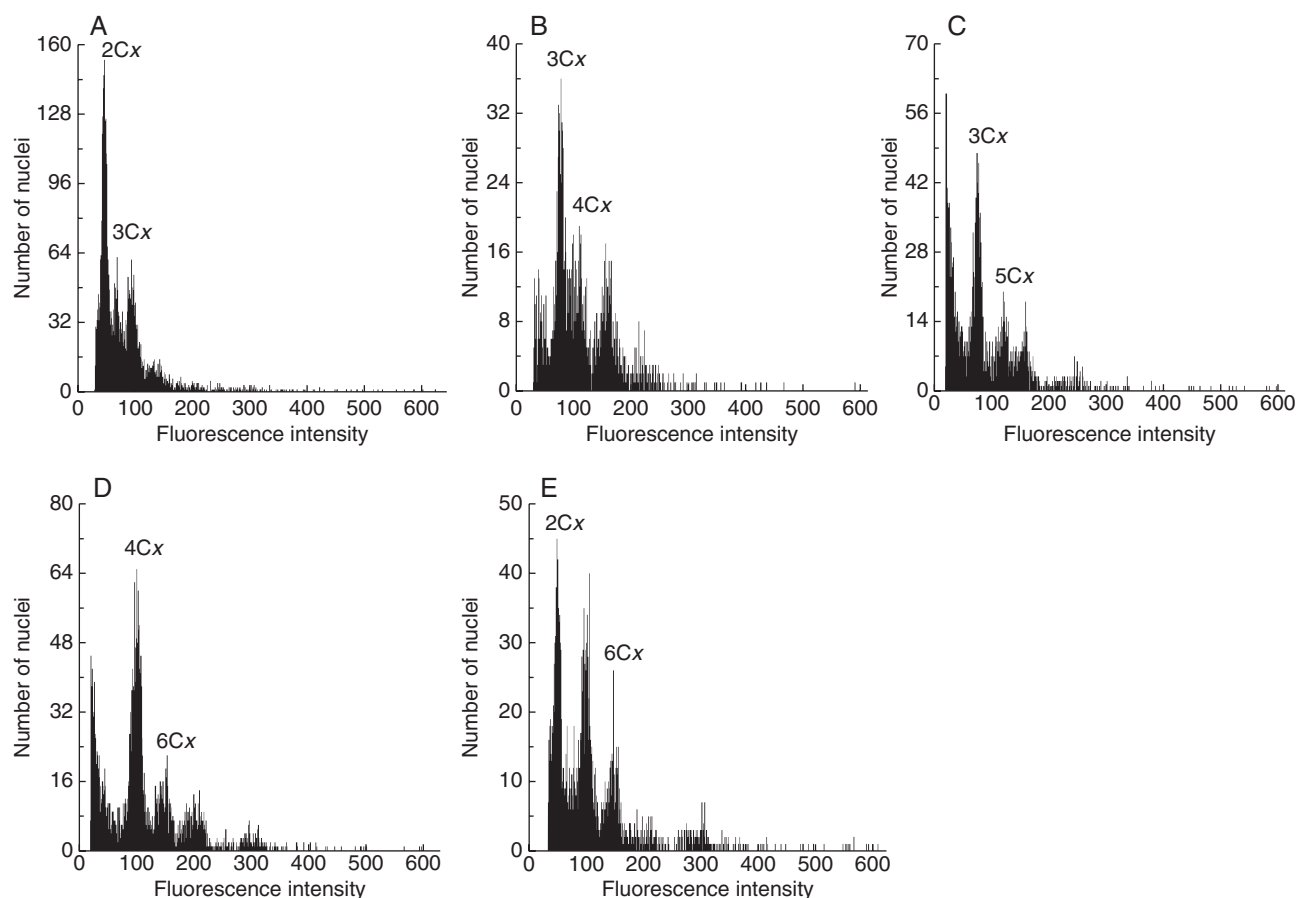
FIG. 2. Cytometric histograms of individual seeds showing different DNA embryo:endosperm ratios. (A) Histogram of a seed showing a 2:3 DNA ratio corresponding to the double fertilization of a meiotic embryo sac by a reduced sperm cell from a diploid (fluorescence intensity $2Cx$, 45·46; $3Cx$, 67·11, peak index: 0·68). (B) Histogram displaying a seed with a 3:4 DNA ratio generated by the double fertilization of a meiotic embryo sac by reduced sperm cells from a tetraploid (triploid B_{II}) (fluorescence intensity $3Cx$, 76·00; $4Cx$, 105·78, peak index: 0·72). (C) Histogram showing a seed with a 3:5 DNA ratio, derived from the double fertilization of an aposporous embryo sac by a reduced sperm cell from a diploid (S_{III}) (fluorescence intensity $3Cx$, 74·74; $5Cx$, 122·80; peak index: 0·61). (D) Histogram with a 4:6 DNA ratio obtained from the double fertilization of an aposporous embryo sac by a reduced sperm from a tetraploid (B_{III}) (fluorescence intensity $4Cx$, 99·74; $6Cx$, 147·58; peak index: 0·67). (E) Histogram of a seed with a 2:6 DNA ratio, derived from the parthenogenetic development of the egg cell of an aposporous embryo sac and fertilization of the polar nuclei by a reduced sperm cell from a tetraploid donor (M) (fluorescence intensitiy $2Cx$, 49·45; $6Cx$, 147·04; peak index: 0·34). Cx denotes monoploid DNA content. The mean value of florescence intensity and peak indexes for different DNA content are described in Supplementary Data Tables S1 and S2.

Burton and Hanna, 1992). Seed recovery and cross-hybridization after interploidy crosses was also documented in the apomictic species *Ranunculus auricomus* (Horandl and Temsch, 2009).

Analysis of the seed formation pathways showed that in open pollination all genotypes develop seeds through the normal sexual reproduction mode (i.e. the double fertilization of a meiotic embryo sac by reduced male gametes). Similar results were obtained after experimental crosses between two diploid genotypes of the species by Siena *et al.* (2008). On the other hand, conspecific interploidy crosses and interspecific interploidy-induced self-pollination provided evidence of variations in the functionality of aposporous embryo sacs and the capacity for apomixis among genotypes. When exposed to pollen from higher ploidy, all individuals from populations R2 and R6 formed seeds exclusively by selfing, but three genotypes from population R5 formed seed by different reproductive pathways involving both meiotic and aposporous embryo sacs. Besides seeds produced by selfing, these plants were able to form seeds after the double fertilization of aposporous embryo sacs with pollen from either the diploid or the tetraploid donors

and from the double fertilization of meiotic sacs with pollen from the tetraploid donor. Thus, triploid (with 3:4 and 3:5 embryo:endosperm DNA ratios) and tetraploid (with 4:6 embryo:endosperm DNA ratio) seeds were obtained. The recovery of triploid individuals in experimental $2x \times 4x$ mating was also reported by Norrmann *et al.* (1994) in the species after *in vitro* ovary rescue followed interploidy intraspecific crosses. Moreover, triploid individuals were detected in natural populations of other species of the genus such as *P. quadrifarium*, *P. notatum*, *P. intermedium*, *P. simplex* and *P. denticulatum* (Burton, 1942; Quarin *et al.*, 1989; Urbani *et al.*, 2002; Sartor *et al.*, 2011). The generation of new polyploids from diploids indicated that diploid genotypes are not reproductively isolated from the tetraploids and suggested that gene flow between both cytotypes would be frequent in mixed populations of diploid and tetraploid cytotypes. In addition, plant R5#49 formed three seeds by the complete apomictic pathway (i.e. apospory, parthenogenetic development of the embryo, and pseudogamy). Remarkably, this genotype (R5#49) showed the capability to form seeds through five different routes, i.e. (1)

TABLE 2. *DNA embryo:endosperm ratios of seeds derived from diploid genotypes of* P. rufum *fertilized with pollen of 4*x P. rufum *and 4*x P. urvillei, *determined by the FCSS method*

Population	Individual	Pollen source	No. of seeds							Apomixis (%)
			Total	Embryo endosperm ratio						
				2:3	3:4	3:5	4:6	2:5	2:6	
R2	#5	4x P. rufum	29	29						
	#8		40	40						
	#15		21	21						
	#42		14	14						
	#51		14	14						
R5	#13	4x P. rufum	17	15		1	1			
	#22		120	120						
	#38		6	6						
	#49		94	39	22	12	21			
	#51		15	13		1	1			
	#22	4x P. urvillei	21	21						
	#49		20	17					3	15
	#51		3	3						
R6	#13	4x P. rufum	10	10						
	#18		47	47						
	#45		108	108						
	#53		66	66						
	#61		13	13						
	#45	4x P. urvillei	100	100						
	#53		5	5						
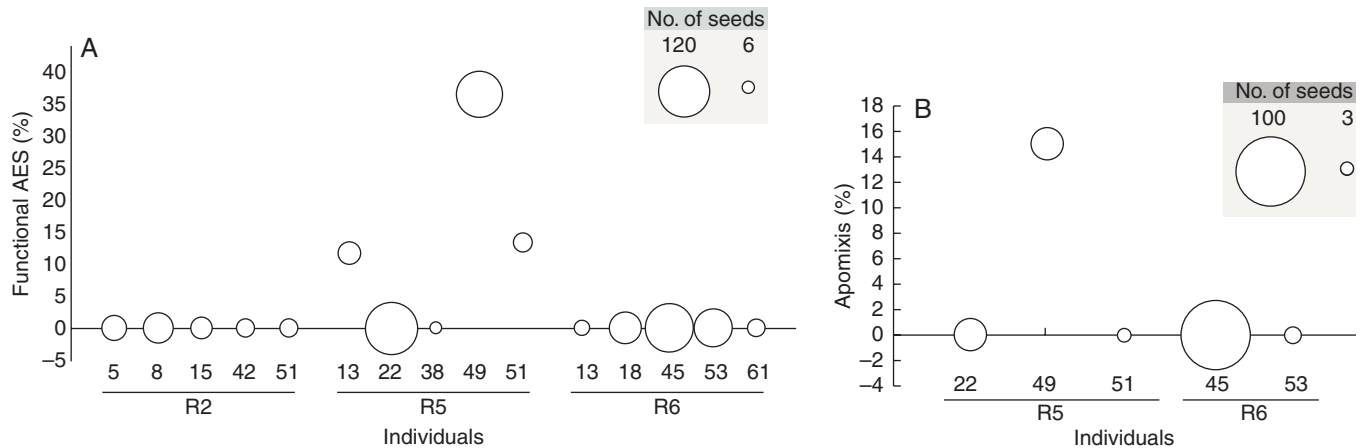
FIG. 3. Plots depicting the functionality of aposporous embryo sacs (AES) and the capacity for apomixis in different diploid genotypes of *P. rufum*. Percentages of seeds derived from functional aposporous embryo sacs after: (A) conspecific interploidy pollination with tetraploid *P. rufum*; (B) induced self-pollination with mentor pollen of tetraploid *P. urvillei*. Circle areas are proportional to the number of caryopses analysed in each genotype.

normal sexuality (diploid progeny, either B_{II} or S_{II}); (2) double fertilization of a meiotic embryo sac with reduced pollen from a tetraploid (triploid B_{II} progeny); (3) double fertilization of an aposporous embryo sac with self pollen (triploid S_{III} progeny); (4) double fertilization of an aposporous embryo sac with reduced pollen from a tetraploid donor (tetraploid B_{III} progeny); and (5) apomixis, i.e. parthenogenesis and pseudogamy of an aposporous embryo sac (diploid M progeny) (Table 2). These abilities are in agreement with previous findings in other species of the genus, indicating that apomictic genotypes are insensitive to the endosperm balance number, and thus they can develop seeds independently of the ploidy level of the pollen donor or even the species (Burton,

1948; Quarin, 1999). Accordingly, the different seed developmental pathways, including apomixis, coexist in some genotypes, and the relative incidence of each one relies on the available pollen source.

Observation of embryo sacs at anthesis of individuals with contrasting reproductive behaviours showed that regardless of the type of progeny produced, all genotypes analysed had the ability to develop aposporous embryo sacs. Thus, the capacity for apospory was not strictly associated with the production of apomeiotic seeds. Moreover, two genotypes from population R5 (R5#13 and R5#51) generated B_{III} hybrids, but not maternal progeny. These results indicated that although aposporous embryo sacs were functional in these genotypes, parthenogenesis

TABLE 3. *Cytoembryological analyses of flowers at anthesis of* P. rufum *diploid genotypes*

Population*	Individual	No. of scored ovules	Proportion of ovules bearing:			
			AbES	MES	AES	MES + AES
R2	#5	55	34·5	63·6	0	1·8
	#51	60	16·7	80·0	0	3·3
R5	#49	123	28·4	58·5	0·8	12·2
R6	#18	54	48·1	38·9	0	13·0
	#45	104	3·8	90·4	0	5·8
	#53	85	49·4	40·0	2·3	8·2

Embryo sac types: AbES, aborted; MES, meiotic; AES, aposporous.
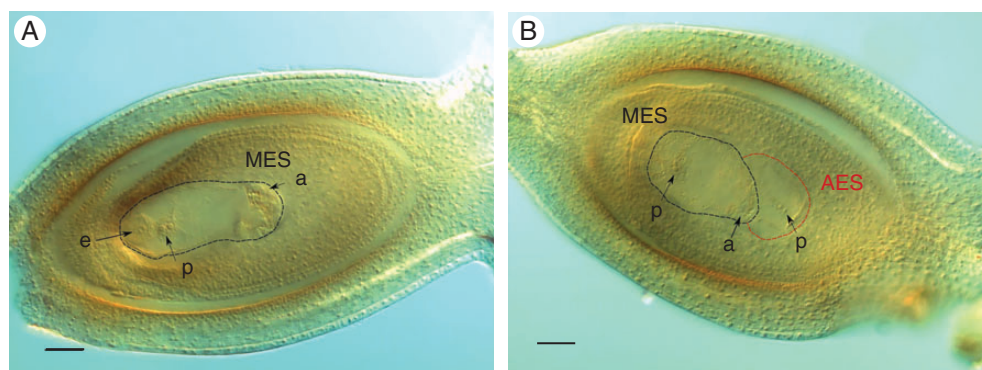*According to Sartor *et al.* (2011).

FIG. 4. Cytoembryological analysis of diploid plants of *Paspalum rufum*. (A) Ovule with a single meiotic embryo sac from plant R6#45. (B) Ovule bearing a meiotic sac plus an aposporous-like embryo sac of plant R5#49. MES, meiotic embryo sac; AES, aposporous-like embryo sac; p, polar nuclei; e, egg cell; s, synergids; a, antipodals. Scale bars = 150 μm.

might be the limiting point for apomictic reproduction. Thus, apospory and parthenogenesis might be uncoupled in some genotypes. Moreover, parthenogenesis could be affected by the pollen source. Recently, in an extended survey of the reproductive behaviour in natural populations of different *Paspalum* species, B$_{III}$ seeds were detected in different populations of *P. alcalinum*, *P. denticulatum*, *P. nicorae* and *P. rufum* (although at higher ploidy levels), indicating that apospory and parthenogenesis may be uncoupled in these species (Sartor *et al.*, 2011). In *Poa pratensis* it was proposed that parthenogenesis is contingent on apospory but not vice versa, and that their traits are controlled by distinct genetic factors (Albertini *et al.*, 2001; Matzk *et al.*, 2005). Separation between both apomictic components was also documented for *R. auricomus* (Horandl and Temsch, 2009).

Interestingly, in conspecific interploidy crosses, the genotype R5#49 produced up to 30·35 % of the total caryopses from aposporous embryo sacs, but, according to cytoembryological observations, only 12 % of the ovules contained aposporous sacs. This difference suggested that the development of aposporous sacs prevails over that of meiotic sacs when pollen from a higher ploidy level is present and compatible pollen of the same ploidy level is absent. In a recent work on tetraploid facultative apomictic *P. malacophyllum*, it was found that different accessions gave rise to plants mostly via the apomictic reproductive pathway, although at the initial developmental stages functional megaspores and meiotic embryo sacs were observed (Hojsgaard *et al.*, 2013). The authors concluded that at early stages both

reproductive routes were developmentally unstable, but apomixis was progressively stabilized through the developmental programme under the proper (epi)genetic background at the polyploid level. Accordingly, in diploid *P. rufum*, both types of embryo sacs coexist until anthesis. It could be possible that in some genotypes, such as R5#49, the presence of pollen from a higher ploidy favours aposporous development (as in a polyploid environment). Conversely, in other genotypes such as R6#45 and R6#53, a failure of some of the components of apomictic reproduction might cause the developmental pathway involving the aposporous embryo sac to decline, favouring the meiotic sac. Accordingly, the potential of the diploid cytotypes to complete the apomictic pathway would be enhanced with the increment of the ploidy. Although apomixis capacity is present at the diploid level in specific genotypes, as was also reported by Siena *et al.* (2008), the fertilization of non-reduced embryo sacs would stimulate the formation of apomeiotic seed from them. This behaviour may explain, at least in part, the strong association between apomixis and polyploidy, and the fact that after duplication of chromosomes of a diploid sexual, apomictic tetraploid plants are recovered (Quarin *et al.*, 2001). These findings suggest that genetic determinants of apomixis present in diploids are not sufficient for an appreciable expression of the trait. The action of other factors related to gene expression or regulation must be affecting their function. The results presented in this work demonstrate the existence of variation in the functionality of a component of apomictic reproduction in diploid

cytotypes of *P. rufum* and detect genotypes with contrasting reproductive modes. Genotypes identified here (such as R6#45 and R5#49) can be crossed to generate segregating populations for studying the apomixis determinants at the diploid level. Moreover, analysis of their expression patterns, the quantification of their transcript levels and the understanding of their regulation mechanisms could help to design new strategies for recreating apomixis in a diploid genome environment.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford journals.org and consist of the following. Table S1: mean values of fluorescence intensity of seeds from diploid *P. rufum* determined by the FCSS method. Table S2: average peak indexes of *P. rufum* seeds derived from different seed formation pathways. Figure S1: box and whisker plot of fluorescence intensity determined by the FCSS method.

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