BIOLOGIA PLANTARUM 55 (2): 391-395, 2011

BRIEF COMMUNICATION

Residual sexuality and its seasonal variation in natural apomictic *Paspalum notatum* accessions

R.N. REBOZZIO, M.E. SARTOR, C.L. QUARIN and F. ESPINOZA*

Instituto de Botánica del Nordeste, CONICET, Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste, Sargento Cabral 2131 (3400), Corrientes, Argentina

Abstract

Traditionally, tetraploid *Paspalum notatum* was considered an obligate or a facultative apomict according to cytoembryological analyses. The degree of facultativeness was usually determined by the relative amount of mature ovules bearing aposporous or meiotic (sexual) embryo sacs, or both together. We established, through progeny tests conducted with the aid of AFLP markers, the degree of residual sexuality expressed in four selected biotypes. The results showed it to be substantially and significantly lower than predicted by previous embryological analyses for the same biotypes. Moreover, the lowest expression of residual sexuality was coincident with maximum flowering period. Seed development in facultative apomictic *P. notatum* shows a definite bias against meiotic embryo sacs.

Additional key words: AFLP markers, facultative apomixis, bahiagrass.

An important proportion of species in the grass genus *Paspalum* represent agamic complexes composed of sexual diploid and apomictic polyploid counterparts (Quarin 1992). Apomixis, asexual reproduction by seeds (Nogler 1984), is characteristic of most polyploid species or races in the genus. The mode of apomixis is usually apospory followed by parthenogenesis. *Paspalum notatum* Flügge, a perennial rhizomatous forage grass distributed from Mexico to Argentina in South America, comprise diploid (2n=2x=20), triploid (2n=3x=30), tetraploid (2n=4x=40) and pentaploids (2n=5x=50) cytotypes (Gates *et al*. 2004). The most common biotype throughout the natural distribution of the species is tetraploid, apomictic, pseudogamous, and self-fertile (Burton 1948). Diploid cytotypes, which reproduce sexually and are allogamous due to a self-incompatibility system (Burton 1955), originally occupied a limited area in South America (Burton 1967, Daurelio *et al*. 2004). Diploid and tetraploid forms are now widely cultivated throughout the southern USA regions. Triploid and pentaploid individuals are apomictic and occur sporadically in wild populations (Gates *et al*. 2004). Early studies based on experimental crosses and progeny tests (Burton 1948) indicated that tetraploid races were obligate apomicts. However, detailed embryological studies carried out on a wide-ranging collection of *P. notatum* indicated that most tetraploid genotypes from different origins showed residual sexuality (Martínez *et al*. 2001, Espinoza *et al*. 2006). These authors observed that a varying proportion of mature ovules, depending upon the considered genotype, showed a meiotic (sexual) embryo sac. The meiotic sac could be alone or accompanied by one or several aposporous sacs in the same ovule. The eventual fertilization of meiotic sacs would lead to sexual reproduction. Thus, in spite of the genetic capacity for apomixis, most tetraploid genotypes

Received 19 February 2010, *accepted* 11 May 2010.

Abbreviations: AFLP -amplified fragment length polymorphism; EF - end of flowering season; FCSS - flow cytometric seed screen; m - maternal; MF - stage of maximum flowering; p - paternal.

Acknowledgements: We thank Juan César Vilardi and Andrea Panseri for their technical assistance and Florencia Galdeano who managed the flow cytometer. We also thank Henry A. Fribourg and Michael Hayward for their kindly assistance concerning idiomatic English. This work was supported by Agencia Nacional de Promoción Científica y Técnica (grants PICT 13578 and PAV 137/3), Secretaría General de Ciencia y Técnica, Universidad Nacional del Nordeste and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Quarin and Espinoza belong to the research staff of CONICET. Rebozzio and Sartor received fellowships from CONICET.

* Corresponding author; fax: (+54) 3783 427131, e-mail: espinoza@agr.unne.edu.ar

showed a residual ability for sexual reproduction. According to embryological data reported previously (Martínez *et al*. 2001, Espinoza *et al*. 2006), this facultative capacity might be responsible for a sexual origin of a high proportion of individuals in the progenies of some specific genotypes. However, the lack of visual morphological variations in progeny tests conducted by Burton (1948) would indicate that the ultimate expression of residual sexuality is less significant than that suggested by embryological analyses. On the other hand, variation in the degree of apomictic expression was observed to correlate with different stages of flowering in *P. cromyorrhizon*, a close relative of *P. notatum* (Quarin 1986). Formerly, embryological analysis of the mother plant and morphological screening of aberrant progenies were the traditional procedures employed for assessing the mode of reproduction in grasses (Young *et al*. 1979, Burson and Bennett 1970, Norrmann *et al*. 1989, Martínez *et al*. 2001, Espinoza *et al*. 2006). Cytoembryological analysis is still the most informative method to describe the processes involved in apomictic reproduction. At the present time, progeny tests conducted with the aid of molecular markers may rapidly assess the quantitative apomictic expression of a given plant by the proportion of the progeny bearing the maternal genotype, while the non-maternal individuals represent the proportion of sexual reproduction.

The embryo:endosperm relative DNA content, established by flow cytometric seed screen analysis (FCSS) (Matzk *et al*. 2000), is a particularly interesting method to determine in *P. notatum* the proportions of seed that a facultative apomictic plant produces by sexual events or by apomixis. In angiosperms with sexual reproduction, a seed consists of a 2n embryo and 3n endosperm bearing maternal (m) to paternal (p) ratios of 1m:1p and 2m:1p, respectively. Any deviation in the endosperm ratio seriously affects the seed development. Apomictic species usually maintain this requirement in different ways. For example, aposporous apomict pseudogamous *Panicum maximum* conserved the 2m:1p ratio, as only one, unreduced polar nucleus contributes from the female while fertilization provides a reduced male gamete (Savidan 2000). The 2m:1p endosperm ratio is conserved in apomictic pseudogamous triploid *Boechera holboellii* in a different way, since only unreduced female and male gametes participate in a viable seed development (Taskin *et al*. 2009). In contrast, most apomicts in *Paspalum*, including *P. notatum*, form endosperms with a 4m:1p ratios. Thus, a mature seed developed by sexual means has a 2:3 embryo:endosperm DNA content ratio, while a seed which originated by apospory, parthenogenesis and pseudogamy shows a 2:5 embryo:endosperm ratio.

The objectives of this research were: *1*) to determine the effective residual sexuality expressed in progenies of apomictic tetraploid genotypes of *P. notatum* using AFLPs markers, in comparison with sexuality expressed at flowering (embryological results); and *2*) to investigate whether the quantitative expression of sexuality has different values in these facultative apomicts at maximum flowering and the end of the flowering cycle.

Three tetraploid plants of *Paspalum notatum* Flügge collected in Brazil (Q4008, Q4012, and Q4023) and one from Argentina (Q3778) were selected as mother plants. The four accessions were initially collected as rhizomes of single plants and space-planted 1 m apart in a field nursery at Corrientes, Argentina. The reproductive mode of each selected genotype had been characterized previously by cytoembryological techniques (Martínez *et al*. 2001, Espinoza *et al*. 2006). Seeds formed in open-pollinated conditions were harvested from the four selected genotypes to generate different progenies. Seeds were germinated in sterilized soil, the seedlings were grown in individual pots containing 500 cm^3 of soil, and then space-planted 0.8 m apart into the field. A first set of four progenies was established from seed formed at the stage of maximum flowering (MF), which occurred at the end of December 2006 and beginning of January 2007. The first set was used to perform the progeny tests with molecular markers and for comparison with results previously obtained through cytoembryological techniques. A second set of four progenies was simultaneously established from seed produced at the end of the flowering period (EF), March 2007. Both sets of progenies were then used to compare the residual sexual reproduction rates in connection with their MF or EF origins.

The reproductive mode of each accession was analysed through progeny tests conducted with AFLP markers. It was assumed that all those individuals showing the same fingerprint pattern as the parental genotype had originated by apomixis (apospory + parthenogenesis), while all those individuals of the offspring that showed a non-maternal fingerprint pattern were assumed to have originated from a sexual event. Genomic DNA was isolated from fresh leaf tissue using the general procedures of the protocol described by Dellaporta *et al*. (1983). The AFLP procedure was undertaken following the manufacturer's instructions of the AFLP *Analysis System I* (*Invitrogen*, Carlsbad, USA). The 38 primer combinations were screened on parental plants: Q3778, Q4008, Q4012 and Q4023, and five of them (E31M33, E31M34, E34M35, E34M37 and E34M39) were selected because they showed clear and recordable bands appropriate to compare the fingerprint patterns. The total number of bands considered for these primers combinations varied between 206 and 330, depending on the progeny. The ploidy level of non-maternal plants (sexual origin) was determined by flow cytometry (*Partec* ploidy analyzer *PA-II,* Germany) in leaf tissue. The flow cytometric seed screen (FCSS) method described by Matzk *et al*. (2000) was used as a control to confirm the results of progeny tests conducted by molecular markers in one of the four accessions analyzed (Q4023). A χ^2-test was used to determine the goodness of fit between the expected results according to previous cytoembryological data and the residual sexuality determined by progeny tests with AFLP

markers. A Monte Carlo test was performed in order to ascertain the association between the degree of sexual expression and the flowering phases in which the seeds were formed. The method has been implemented using CLUMP program which is freely available from http://www.smd.qmul.ac.uk/statgen/dcurtis/software.html.

Results showed that progenies of genotypes Q4008 and Q4012 from seeds generated at stages of maximum flowering displayed uniform fingerprint patterns identical to band patterns of their parental genotypes. 46 descendants of Q3778 were analyzed and only two showed non-maternal fingerprints, and similarly there were two non-maternal plants among the 39 descendants analyzed for Q4023 (Table 1). Flow cytometric analysis showed that these four aberrant plants had the same ploidy level as the corresponding parental plant. This indicated that they originated in a meiotic embryo sac from a normal n+n sexual fertilization event, and excluded the possibility of a 2n+n event (fertilization of an unreduced egg cell in an aposporous embryo sac by a reduced sperm nucleus). The genotype Q4012, an obligate apomict according previous cytoembryological analyses, showed in its whole progeny the same band pattern as the parental fingerprints. The degree of sexuality expressed in the progenies of Q3778 and Q4023 and the lack of sexual expression in the progeny of Q4008 differ substantially and significantly from the proportion of sexual reproduction that could have been predicted by previous embryological analyses in these facultative apomictic genotypes (Table 1). These results suggest an extraordinary advantage for seed development in aposporous embryo sacs to the detriment of seed development in meiotic sacs. This advantage is most likely sustained in the genetic ability for precocious seed development observed in apomictic plant species (Curtis and Grossniklaus 2008). In this way, Savidan (1982) reported a clear difference of timing between the meiotic *versus* the aposporous pathways of development in *Panicum maximum*: most aposporous embryo sacs of apomictic plants had long reached maturity by the time of anthesis while in sexual plants only 2/3 of the ovules had a fully mature developed embryo sac in this stage. In agreement with these results in *Panicum maximum,* Martínez *et al*. (1994) observed in pseudogamous apomictic tetraploid *P. notatum* that 2n+n progenies were produced, instead of maternal progenies, when pollination was artificially shifted two or three days prior to anthesis. Precocious seed development was also documented in many apomictic *Paspalum* species, since proembryos were repeatedly observed by the time of

Table 1. Potential and real expression of apomixis in different *Paspalum notatum* accessions. Progenies established from seed harvested at the time of maximum flowering. n - number. * - according previous embryological data of Martinez *et al*. (2001) and Espinoza *et al*. (2006).

Accession	Seed set [%]	Number of plants per progeny	Observed degree of sexuality [%]	Expected sexual reproduction [%]* minimum	maximum	χ^2	*P*
Q3778	37.7	46	4.34	23.5	32.6	14.78	<0.001
Q4008	26.8	50	0.00	15.5	35.5	15.78	<0.001
Q4012	41.5	44	0.00	0.0	0.0	-	-
Q4023	38.6	39	5.12	35.7	95.2	65.80	<0.001

Table 2. Comparative progeny tests of four tetraploid *P. notatum* genotypes with regard to the phase of flowering (MF - full flowering period, EF - end of the flowering season). Tests were conducted with AFLP markers using genomic DNA of leaf-blades (lb), and confirmed for genotype Q4023 by flow cytometry in single seed (ss) relative embryo:endosperm DNA contents. Significance of differences among MF and EF were calculated with Barnard's Monte Carlo test: NS - non significant, * - significant and ** - highly significant.

Parental genotype	Flowering	Number of plants per progeny	Number of plants with sexual origin	apomictic origin	χ^2	$P < 0.05$
Q3778 (lb)	MF	46	2	44	1.6544	0.3676 NS
	EF	33	4	29		
Q4012 (lb)	MF	44	0	44	6.3371	0.0160 *
	EF	37	5	32		
Q4008 (lb)	MF	50	0	50	6.3830	0.0260 *
	EF	50	6	44		
Q4023 (lb)	MF	39	2	37	0.3964	0.7103 NS
	EF	59	5	54		
Q4023 (ss)	MF	41	3	38	1.1233	0.4855 NS
	EF	41	6	35		
Overall					11.8300	0.0020 **

anthesis in aposporous embryo sacs (Burson and Bennett 1970, 1971, Espinoza *et al*. 2001). Thus, the precocious seed development starting with early embryo development in aposporous sacs may account for the remarkably higher proportion of progenies raised by means of apomixis than the expected values according the observed proportion of ovules bearing one meiotic sac at flowering, or one meiotic sac accompanied by aposporous embryo sacs.

A second set of progenies was generated at the end of the flowering season from seeds of the same four genotypes: Q3778, Q4008, Q4012, and Q4023. Progeny tests with AFLP markers indicated that all genotypes produced some aberrant individuals among these EF progenies (Table 2). Flow cytometric analysis in leaf tissue showed that all aberrant descendants of Q3778, Q4008, and Q4012 had the same ploidy level as the parental plant (tetraploid) and therefore all originated from sexual events in meiotic sacs (n+n). Otherwise, the EF progeny of Q4023 exhibited seven non-maternal plants from which only five were considered to have a sexual n+n origin, since flow cytometry indicated that two aberrant plants were hexaploid and had originated by fertilization in an aposporous embryo sac (2n+n). Aberrant plants observed in all EF progenies indicated that the four genotypes, included Q4012 that was previously classified as an obligate apomict, showed some degree of residual sexual reproduction at least at the onset of the flowering season (Table 2). The results from these four genotypes suggest that tetraploid *P. notatum* could be considered a facultative apomictic species. On the other hand, the four genotypes showed a higher ability to produce aberrant plants among EF progenies than among MF progenies. The proportions of non-maternal plants in progenies of Q4012 and Q4008 were significantly correlated with the flowering stages of seed formation (Table 2), while the EF progenies of Q3778 and Q4023 showed a higher rate of non-maternal plants than their MF progenies, even though the observed values were not significantly different. However, there is a general tendency to increase the residual sexuality rate in apomictic tetraploid *P. notatum* at the end of the summer, when the environmental conditions that promote flowering are less favourable. The highest values for asexual reproduction are coincident with the phase of flowering when the plant is producing the greatest proportion of its seed.

As a control for our general data, two different methods were used to test the progenies of Q4023: AFLP markers performed with DNA obtained from leaf tissue of its progeny (as done with the other three accessions), and flow cytometry in single seed samples derived from open pollination of Q4023. 41 seeds from each flowering phase (MF and EF) were analyzed. Most MF seeds (38 out of 41) showed a 2:5 embryo:endosperm DNA content ratio indicative of apomictic formation, while the remaining three seeds had a 2:3 ratio as typical of sexual development. When 41 seeds of the EF period were analyzed, three different results were observed: most seeds (31) proved to be originated by apomixis since the embryo:endosperm DNA content ratio was 2:5, while observed 2:3 ratios revealed that six other seeds had developed by sexual means. Interestingly, there were 4 remaining seeds which exhibited a embryo:endosperm ratio 3:5. These most likely represents hexaploid B_{III} hybrids which originated by fertilization of unreduced egg cells of aposporous embryo sacs. Though these seeds arose from an ultimate sexual event (2n+n), in fact they developed from aposporous embryo sacs. Certainly, apospory is the first step in the expression of apomixis and so, for the final analysis, we arbitrarily added these 4 seeds to the 31 developed through completely apomictic pathways. Both kinds of progeny tests showed equivalent values of residual sexuality (Table 2), regardless of the method that we employed: flow cytometry in seed progenies or AFLP markers in plant progenies. This similarity indicates that the results of progeny tests conducted by flow cytometry seed screen are equivalent to results obtained by molecular markers employed in fully developed progeny plants.

The low efficiency of meiotic sacs to produce progenies through sexual events may also be ascribed to meiotic irregularities that have been observed by several authors in different tetraploid strains of *P. notatum* (Magoon and Manchanda 1961, Fernandes *et al*. 1973, Stein *et al*. 2004). Cytologically unbalanced male and female gametes may lead to pre- or post-zygotic abortion. In fact, all four accessions analyzed here showed seed set values below 50 %. This means that more than 50 % of the spikelets failed to form grain (caryopsis). Our results suggest that most seed was formed from those ovules carrying aposporous embryo sacs. The final expression of the apomictic trait in tetraploid *P. notatum* depends upon the competition between aposporous and meiotic embryo sacs, a definite bias against sexuality.

The quantitative expression of sexuality in a constitutive versatile apomict may show significantly divergent values when analyzed at different phases of the reproductive plant cycle. The potential for sexuality evaluated at the flowering phase by cytoembryological analyses show higher values than the functional sexuality observed at seed phase by flow cytometric data or in established progenies through the detection of non-maternal fingerprint patterns by molecular markers.

Our observation that the level of functional sexuality in facultative apomicts is significantly lower than observed through cytoembryological analysis and the observations that the frequency of apomixis has a propensity to increase at maximum flowering are two issues of particular implication in facultative apomictic tetraploid races of *P. notatum*. There is a definite trend toward apomixis. However, residual sexuality may act as an inner reserve for developing new genetic variants when less favourable environmental conditions occur.

References

Burson, B.L., Bennett, H.W.: Cytology, method of reproduction, and fertility of Brunswickgrass, *Paspalum nicorae* Parodi. - Crop Sci. **10**: 184-187, 1970.

Burson, B.L., Bennett, H.W.: Chromosome numbers, microsporogenesis, and mode of reproduction of seven *Paspalum* species. - Crop Sci. **11**: 292-292, 1971.

Burton, G.W.: The method of reproduction in common bahiagrass, *Paspalum notatum*. - J. amer. Soc. Agron. **40**: 443-452, 1948.

Burton, G.W.: Breeding pensacola bahiagrass. *Paspalum notatum*: I. Method of reproduction. - Agron. J. **47**: 311-314, 1955.

Burton, G.W.: A search for the origin of pensacola bahiagrass. - Econ. Bot. **21**: 379-382, 1967.

Curtis, M.D., Grossniklaus, U.: Molecular control of autonomous embryo and endosperm development. - Sex. Plant Reprod. **21**: 79-88, 2008.

Daurelio, L.D., Espinoza, F., Quarin, C.L., Pessino, S.C.: Genetic diversity in sexual diploids and apomictic tetraploid populations of *Paspalum notatum* situated in sympatry or allopatry. - Plant Syst. Evol. **244**: 189-199, 2004.

Dellaporta, S.L., Wood, J., Hicks, J.B.: A plant DNA minipreparation: Version II. - Plant mol. Biol. Rep. **1**: 19-21, 1983.

Espinoza, F., Urbani, M.H., Martínez, E.J., Quarin, C.L.: The breeding system of three *Paspalum* species with forage potential. - Trop. Grassland **35**: 211-217, 2001.

Espinoza, F., Daurelio, L.D., Pessino, S.C., Valle, E.M., Quarin, C.L.: Genetic characterization of *Paspalum notatum* accessions by AFLP markers. - Plant Syst. Evol. **258**: 147-159, 2006.

Fernandes, M.I.B.M., Barreto, I.L., Salzano, F.M.: Cytogenetic, ecologic and morphologic studies in Brazilian forms of *Paspalum notatum.* - Can. J. Genet. Cytol. **15**: 523-531, 1973.

Gates, R.N., Quarin, C.L., Pedreira, C.G.S.: Bahiagrass. - In: Moser, L.E., Burson, B.L., Sollenberger, L.E. (ed): Warm-season (C4) Grasses. Pp 651-680. ASA, CSSA, and SSSA Publishers, Madison 2004.

Magoon, M.L., Manchanda, P.L.: A cytological study of some species in the genus *Paspalum*. - Indian J. Genet. Plant Breed. **21**: 212-220, 1961.

Martínez, E.J., Espinoza, F., Quarin, C.L.: B_{III} progeny (2n + n) from apomictic *Paspalum notatum* obtained through early pollination. - J. Hered. **85**: 295-297, 1994.

Martínez, E.J., Urbani, M.H., Quarin, C.L., Ortiz, J.P.A.: Inheritance of apospory, *Paspalum notatum*. - Hereditas **135**: 19-25, 2001.

Matzk, F., Meister, A., Schubert, I.: An efficient screen for reproductive pathways using mature seeds of monocots and dicots. - Plant J. **21**: 97-108, 2000.

Nogler, G.A.: Gametophytic apomixis. - In: Johri, B.M. (ed.): Embryology of Angiosperms. Pp. 475-518. Springer-Verlag, Berlin 1984.

Norrmann, G.A., Quarin, C.L., Burson, B.L.: Cytogenetics and reproductive behaviour of different chromosome races in six *Paspalum* species. - J. Hered. **80**: 24-28, 1989.

Quarin, C.L.: Seasonal changes in the incidence of apomixis of diploid, triploid, and tetraploid plants of *Paspalum cromyorrhizon*. - Euphytica **35**: 515-522, 1986.

Quarin, C.L.: The nature of apomixis and its origin in panicoid grasses. - Apomixis Newslett. **5**: 8-15, 1992.

Savidan, Y.: Nature et héredité de l´apomixie chez *Panicum maximum* Jacq. - Ph.D. Thesis, Université de Paris-Sud, Centre d'Orsay, Paris 1982.

Savidan, Y.: Apomixis: genetics and breeding. - Plant Breed. Rev. **18**: 13-86, 2000.

Siena, L.A., Sartor, M.E., Espinoza, F., Quarin, C.L., Ortiz, J.P.A.: Genetic and embryological evidences of apomixis at the diploid level of *Paspalum rufum* support recurrent auto-polyploidization in the species. - Sex. Plant Reprod. **21**: 205-215, 2008.

Stein, J., Quarin, C.L., Martínez, E.J., Pessino, S.C., Ortiz, J.P.A.: Tetraploid races of *Paspalum notatum* show polysomic inheritance and preferential chromosome pairing around the apospory-controlling locus. - Theor. appl. Genet. **109**: 186-191, 2004.

Taskin, K.M., Turgut, K., Scott, R.J.: Apomeiotic pollen mother cell development in the apomictic *Boechera* species. - Biol. Plant. **53**: 468-474, 2009.

Young, B.A., Sherwood, R.T., Bashaw, E.C.: Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. - Can. J. Bot. **57**: 1668-1672, 1979.