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Evaluation of different methods for assessing the reproductive mode of weeping lovegrass plants, *Eragrostis curvula* (Schrad.) Nees

Mauro Meier[A], *Diego Zappacosta*[A], *Juan Pablo Selva*[A], *Silvina Pessino*[B] *and Viviana Echenique*[A,C]

[A]Laboratorio de Biotecnología Vegetal, CERZOS-CONICET, CCT Bahía Blanca, Camino La Carrindanga km 7 and Departamento de Agronomía, Universidad Nacional del Sur, San Andrés 800, 8000 Bahía Blanca, Argentina.
[B]Laboratorio de Biología Molecular, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Parque Villarino, S2125ZAA Zavalla, Provincia de Santa Fe, Argentina.
[C]Corresponding author. Email: echeniq@criba.edu.ar

Abstract. Weeping lovegrass is a forage grass cultivated in semiarid regions of the world that reproduces mainly by apomixis (diplospory), a process that involves the formation of asexual seeds and bypasses the processes of meiosis and fertilisation. The aim of this work was to evaluate and compare different techniques (cytoembryology, callose deposition, flow cytometry and progeny tests) to determine the reproductive mode of weeping lovegrass. Typical sexual and apomictic processes were clearly differentiated using cytoembryology, and different callose deposition patterns were observed in sexual and apomictic genotypes. Previous studies indicated that presence of callose on the cell wall of the megaspore mother cell is associated only with sexual processes. Nevertheless, our results also found callose deposition in apomictic genotypes, although clearly different from the pattern found in sexual processes, allowing discrimination between sexual and apomictic plants. Flow cytometry seed screening using individual seeds did not differentiate between sexual and apomictic plants as the embryo : endosperm DNA content ratio was similar in sexual and apomictic plants. Progeny tests using molecular markers showed uniform patterns in offspring from apomictic plants and variable patterns among the progeny of sexual plants. The results obtained from cytological studies and progeny tests were similar, indicating that both methods provide useful tools for determination of reproductive mode. However, the callose test with aniline blue was faster and easier to use than other techniques.

Introduction

Apomixis is a means of asexual reproduction through seeds that progresses in the absence of meiosis and fertilisation to generate maternally derived clonal progenies (Nogler 1984). Apomixis probably evolved as an alternative reproductive system, through a rearrangement of the developmental programs that constitute the normal sexual pathway (Savidan 2000). Apomictic processes have been classified into three general mechanisms, termed diplospory, apospory and adventitious embryony. Diplospory and apospory are considered to be gametophytic mechanisms, because in both cases formation of an embryo sac is required. Both pathways are initiated during differentiation of the megaspore mother cell (MMC). In diplosporous plants, the MMC fails to enter or to complete meiosis, generating a non-reduced embryo sac through a series of mitoses. In aposporous plants, one or several non-reduced embryo sacs are formed by mitosis from nucellar cells surrounding the MMC. Embryos develop from the unreduced egg cells that are included into these structures. By contrast, adventitious embryony is classified as a sporophytic mechanism, occurring in the absence of megagametophyte formation. It is initiated during ovule development, usually in mature ovules and embryos are initiated by parthenogenesis directly from individual somatic cells from ovule tissues that are external to a sexually derived megagametophyte (Koltunow 1993).

Exploitation of apomixis in major crop plants would provide major benefits to agriculture, including: (i) widespread use and fixation of hybrid vigour, even for those plants for which hybrid production technology is not available; (ii) survival and immediate fixation of combined genetic resources, including wide-cross progeny that are unfit when propagated sexually; (iii) capacity for true seed production from crops that currently propagated by vegetative means; and (iv) access to more rapid breeding programs in response to changing needs and environments (Spillane *et al.* 2004).

Most apomictic species are facultative and can reproduce sexually at a variable level, only a few species being considered as true obligate apomicts. Facultative apomixis confers an environmental and evolutive fitness to some species like Kentucky bluegrass (*Poa pratensis* L.) (Mazzucato *et al.*

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1995). Protocols to evaluate the level of expression of apomixis in experimental or breeding plant materials are useful to assess its potential for variation.

Screening for apomictic reproduction and estimation of incidence level of apomixis in a particular species or genotype is non-trivial. There are several indicators for the occurrence of the trait, such as detection of a high frequency of multiple seedlings, a high rate of seed fertility in plants that are expected to be sterile, and generation of homogeneous progeny (Leblanc and Mazzucato 2001). However, confirmation of the existence of true apomictic events and their rate of occurrence is often an arduous process. In order to accurately and thoroughly phenotype an individual plant for reproductive mode, it is necessary to cytologically examine developmental events that occur in the ovule as well as to characterise genetic variability/uniformity of its progeny. Cytological analysis involves microscopic observation of paraffin- or resin-embedded, sectioned material or cleared organs. Organ clearing is a relatively simple technique compared with sectioning, but must be optimised for the species and the tissues under study, particularly with regard to refractive index of the clearing solution (Ozias-Akins 2006).

Cytoembryological differences are detectable between sexual and apomictic organisms at several developmental steps. Observations to determine the origin of embryo sacs are based on detecting variations in megasporogenesis from the MMC (in the case of diplospory), or nucellar cell initiation (in the case of apospory). In addition, variations in the morphology of embryo sacs are detected during megagametogenesis. Paraffin sectioning methods combined with staining (e.g. safranin-fast green stain, Johansen 1940) have been used over the last century for cytoembryological studies, reproductive development and apomixis research (Leblanc and Mazzucato 2001).

To determine the frequency of functional apomixis with respect to sexual reproduction, progeny analysis tests are necessary. The simplest strategy is to examine the variability/uniformity of progeny produced from the tested plant when pollinated with a genetically divergent male. In this condition, a near-obligate apomictic plant would produce uniform progeny with a genetic constitution reminiscent of itself (Ozias-Akins 2006). In practice, progeny tests are performed with molecular genetic markers which have the advantage of not being affected by age of the plant or the environment. In addition, the number of markers is not a limitation, as a large number of polymorphic bands may usually be detected. Isoenzymes and RAPD (random amplified polymorphic DNA) markers are useful for precocious detection of the mode of reproduction in hybrids, as shown in *Poa pratensis* (Mazzucato *et al.* 1995). RAPD were also successfully applied to the analysis of apomictic reproduction rate in different genotypes of *Paspalum notatum* (Ortiz *et al.* 1997). In tetraploid populations of *Aronia*, the maternal parent and the offspring showed identical RAPD profiles (Persson Hovmalm *et al.* 2004). In the savanna grass, *Hyparrhenia diplandra*, highly variable simple sequence repeat markers were used to demonstrate occurrence of facultative apomixis with rare events of sexual reproduction (Durand *et al.* 2000).

In the case of diplospory, differential patterns of callose deposition during megagametophyte development of sexual and apomictic plants have also been used as a screening tool to identify apomicts (Carman *et al.* 1991; Leblanc *et al.* 1995; Peel *et al.* 1997; Leblanc and Mazzucato 2001; Bicknell and Koltunow 2004). In sexually reproducing plants, the walls of the MMC, the tetrad of megaspores and the degenerating megaspores are differentiated by temporary accumulation of callose. Callose is dissolved from the walls of the selected megaspore during expansion and the initiation of mitotic events of embryo sac development. Meanwhile, apomictic plants are devoid of the callose layer surrounding the MMC (Bhat *et al.* 2005).

To discriminate between apomictic and sexual reproduction, an alternative method is the flow cytometric seed screening (FCSS) test (Matzk *et al.* 2000), which analyses stained embryo and endosperm nucleus of a seed set. In apomictic events, the embryo : endosperm DNA content ratio is often altered, because of the formation of the endosperm from a non-reduced polar nucleus or nuclei. Moreover, polar nuclei/nucleus may be fertilised or not, depending on the species. Comparison of the relative DNA contents between embryo and endosperm nuclei may facilitate deduction of origin of these two tissues (Ozias-Akins 2006). FCSS permits analysis of individual or pooled of seed samples for peaks of nuclear DNA content. Sexual reproduction is expected to result in major $2n$/2C and $3n$/3C peaks from $n+n$ embryos and $2n+n$ endosperm, respectively. Apomictic reproduction leads to a higher variability in DNA content ratios, because of the greater potential for development of $n+0$, $2n+0$, or $2n+n$ embryos and $2n+0$, $2n+n$, or $4n+n$ endosperm (Ozias-Akins and van Dijk 2007).

Eragrostis curvula (weeping lovegrass) is an apomictic tropical perennial grass, originally derived from southern Africa. The type of apomixis present in the *E. curvula* complex is pseudogamous diplospory (Streetman 1963). The complex includes cytotypes with different ploidy levels (e.g. $2x$–$8x$) displaying obligate apomixis, facultative apomixis and sexual reproduction (Voigt and Bashaw 1976; Voigt *et al.* 2004). Weeping lovegrass produces two main types of embryo sacs, namely octanucleated reduced monosporic *Polygonum*-type embryo sacs (Vorster and Liebenberg 1984), and tetranucleated non-reduced diplosporous *Eragrostis*-type sacs, containing an egg cell ($2n$), two synergids ($2n$) and one polar nuclei ($2n$), but lacking antipodals (Crane 2001). The apomictic members of the *E. curvula* complex retain normal meiotic processes for pollen production (Stalker and Wright 1975).

In the last few years, we have been studying the molecular mechanisms involved in *E. curvula* reproduction. Gene expression analysis and genomic methylation patterns were examined in apomictic and sexual genotypes of different ploidy levels (Mecchia *et al.* 2007; Cervigni *et al.* 2008a; Ochogavía *et al.* 2009). A mapping population has also been generated in order to map the locus/loci involved. Phenotypic analysis of our mapping population requires a prior assessment of appropriate methods to analyse the reproductive mode (as sexual or apomictic). Therefore, different techniques (cytoembryology, molecular markers and flow cytometry) were applied to study the reproductive mode of different genotypes of weeping lovegrass that were previously classified as sexual, obligate apomictic and facultative apomictic, with the objective of comparing relative efficiencies to estimate level of apomixis or sexuality observed in this species. Progeny tests and cytoembryological studies have

been concluded to be preferred reference methods to estimate the levels of apomictic as compared with sexual reproduction in this forage grass.

Materials and methods

Plant material

Eleven accessions of weeping lovegrass growing under greenhouse and field conditions (Cabildo, Buenos Aires, Argentina) were evaluated. Four highly polyploid cultivars (Don Pablo $2n = 7x = 70$, Don Eduardo, $2n = 6x = 60$; Don Juan, $2n = 8x = 80$; Kromdraai, $2n = 6x = 60$), two natural tetraploid cultivars (cultivars Tanganyika, $2n = 4x = 40$; OTA-S, $2n = 4x = 40$), two colchicine-induced tetraploid genotypes (UNST1131, $2n = 4x = 40$; UNST1112, $2n = 4x = 40$) obtained by Cardone *et al.* (2006) and two diploid genotypes ($UNST1122_{R0}$, $2n = 2x = 20$; $UNST1122_{R1}$, $2n = 2x = 20$) were analysed.

Cytoembryological studies

Inflorescences were collected at the beginning of anthesis, as which time it was possible to observe all the embryo sac developmental stages, and were fixed in FAA (50% ethanol, 5% acetic acid, 10% formaldehyde and distillated water). Individual spikelets from different panicles were dehydrated in a tertiary butyl alcohol series and embedded in paraplast (Johansen 1940). Samples were sectioned at 10 µm and stained with aniline blue or safranin-fast green. Callose deposition on pistils was visualised by staining with aniline blue (0.005% in buffer K_2PO_4 0.067 M pH 9.5) and observed by using UV microscopy (Carman *et al.* 1991). Observations were carried out with a Nikon Eclipse TE300 light transmission microscope (Tokyo, Japan) or in Nikon Eclipse 80 I with UV2E filter.

Progeny tests

A progeny test was performed on two different sets of genotypes obtained by open pollination from individuals of the apomictic cultivar Tanganyika and the sexual genotype $UNST1122_{R0}$. Progenies were composed of 25 and 20 individual plants, respectively, in a similar way as was reported previously by Daurelio *et al*. (2004) and Matzk *et al*. (2005). Progenies of the facultative apomictic genotypes UNST1131 and UNST1112 were also evaluated (99 and 15 individuals, respectively). In this last case, directed crosses were made using the tetraploid apomictic cultivar Ermelo as pollen donor.

Genomic DNA was extracted from fresh leaf tissue. Plant material was frozen in liquid nitrogen, powdered using mortar and pestle and transferred to 1.5-mL microtubes containing extraction buffer [50 mM TRIS-HCl pH 8, 10 mM EDTA pH 8, 100 mM NaCl, 10% (w/v) SDS and 10 mM β-mercaptoethanol]. The mixture was incubated at 65°C for 20 min, followed by the addition of 200 µL 5 M potassium acetate (KCH_3CO_2) pH 4.8. Samples were incubated on ice for 20 min, and centrifuged for 20 min at 13 000 *g* at room temperature. The supernatant was collected, and one volume of isopropanol was added. Samples were incubated at 10 min at −20°C, and then centrifuged for 4 min at 13 000 *g* at room temperature. Pellets were washed twice with one volume of 70% (v/v) ethanol and resuspended in 50 µL of TE buffer. DNA concentration was determined in a BioRad fluorometer (Hercules, CA, USA) using fluorescent dye Hoechst 33258 (BioRad) and calf thymus DNA as standard.

RAPD DNA experiments were performed by using the protocol described at the CIMMYT Applied Molecular Genetics Laboratory Protocols, CIMMYT, Mexico (http://www.cimmyt.org, accessed 23 March 2011), with appropriate modifications. Four primers (221, 222, 237 and 241) from the NAPS unit list of standard primers were used. Each amplification reaction was performed in a volume of 25 µL containing 1× *Taq* polymerase reaction buffer (Invitrogen, Carlsbad, CA, USA), 10 mM each of deoxynucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 30 ng primer pair combinations, 50 ng genomic DNA template and 1 U *Taq* polymerase (Invitrogen). Amplifications were carried out in a MJ Research thermocycler (Watertown, MA, USA) with the following program: initial denaturation at 94°C for 4 min, 36 cycles of 94°C for 30 s, 36°C for 1 min and 72°C for 1 min, and final extension at 72°C for 5 min. Negative controls were performed by eliminating genomic DNA from the assays. Amplification products were electrophoresed in 6% (w/v) acrylamide gels and silver-stained. Reliability was assessed by the use of duplicates.

Offspring plants were classified as apomictic when RAPD-derived genetic profiles were identical, or exhibited only one polymorphism with respect to the maternal profile and as sexual products when two or more differential features were observed. This consideration was taken into account in order to avoid errors caused by technique artefacts (Arnholdt-Schmitt 2000).

Flow cytometry

Individual seeds obtained by open pollination of the tetraploid cultivar Tanganyika and the diploid genotype $UNST1122_{R0}$ were analysed by FCSS (Matzk *et al.* 2000). For the analysis, suspensions of nuclei from mature seeds were prepared by crushing single dry seeds in Petri dishes with 1 mL of staining buffer [50 mM TRIS-HCl, 2.63 mM $MgCl_2$, 43 mM NaCl, 0.05% (v/v) Triton X-100, 0.01 mM 4'-6-diamidino-2-phenylindole, pH 7.5]. The suspensions were filtered through nylon tissue with 30-µm mesh width. The samples were incubated for 2 min at room temperature before measurements. The fluorescence intensity of around 5000 DAPI-stained nuclei was determined using the Partec PA II flow cytometer (Partec GmbH, Munster, Germany) with the detector operating at 355 nm. Data analysis was performed using FloMax software for the Partec PA II. Ploidy levels were estimated by comparing the DNA peaks of the mentioned plant materials with those of leaves samples from diploid and tetraploid weeping lovegrass plants.

Statistical analysis

To compare the results for cytoembryological studies and progeny tests with molecular markers, Chi-square tests of homogeneity for 3 : 2 contingency tables were used. Non-significant differences among techniques indicated homogeneity.

Results

Cytoembryological studies

In sexual plants stained with aniline blue, the MMC displayed a rectangular shape (Fig. 1*a*), the wall being thickened at the

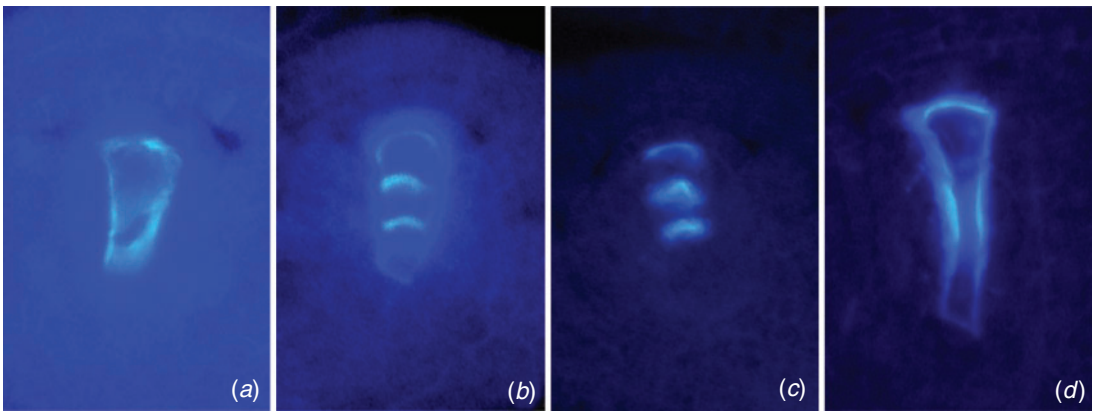

Fig. 1. Callose deposition patterns in sexual ($UNST1122_{R0}$) (*a*–*c*) and apomictic (cv. Tanganyika) (*d*) weeping lovegrass plants. Bar: 10 µm (*a*) megaspore mother cell (MMC). (*b*) Three cells of the linear tetrad of megaspores. (*c*) Degenerated megaspores. (*d*) Elongated megaspore mother cell (EMMC).

micropylar end and completely surrounded by callose. A linear tetrad (Fig. 1*b*), characteristic of meiotic processes, could clearly be observed. The functional megaspore (Fig. 1*c*) was devoid of glucan, while the three callose-covered degenerated megaspores (oriented in the direction of the micropilar pole of embryo) could also be observed.

Callose deposition over the MMC was also observed in apomictic plants, for which no evidence of a typical sexual process was observed (formation of a linear tetrad and later presence of degenerated megaspores). Fluorescence observed on the apomeiotic MMC (Fig. 1*a*) revealed a similar pattern to that observed during sexual development. However, callose deposition observed on elongated megaspore mother cells (EMMC, Fig. 1*d*), showed a characteristic distinctive pattern that in some cases accompanied cell elongation until the first cell division.

Sections stained with safranin-fast green (Fig. 2) confirmed the observations made with aniline blue, and analysis of an increased number of samples through provision of a more detailed analysis of embryo sac development. In spikelets collected from UNST1122, OTA-S and in some pistils of UNST1112, UNST1131 and Kromdraai, typical stages indicative of sexual processes were observed, such as linear tetrads of megaspores (Fig. 2*a*), the three micropylar degenerated megaspores and the functional megaspore (Fig. 2*b*). In addition, binucleated stage (Fig. 2*c*), tetranucleated (Fig. 2*d*)

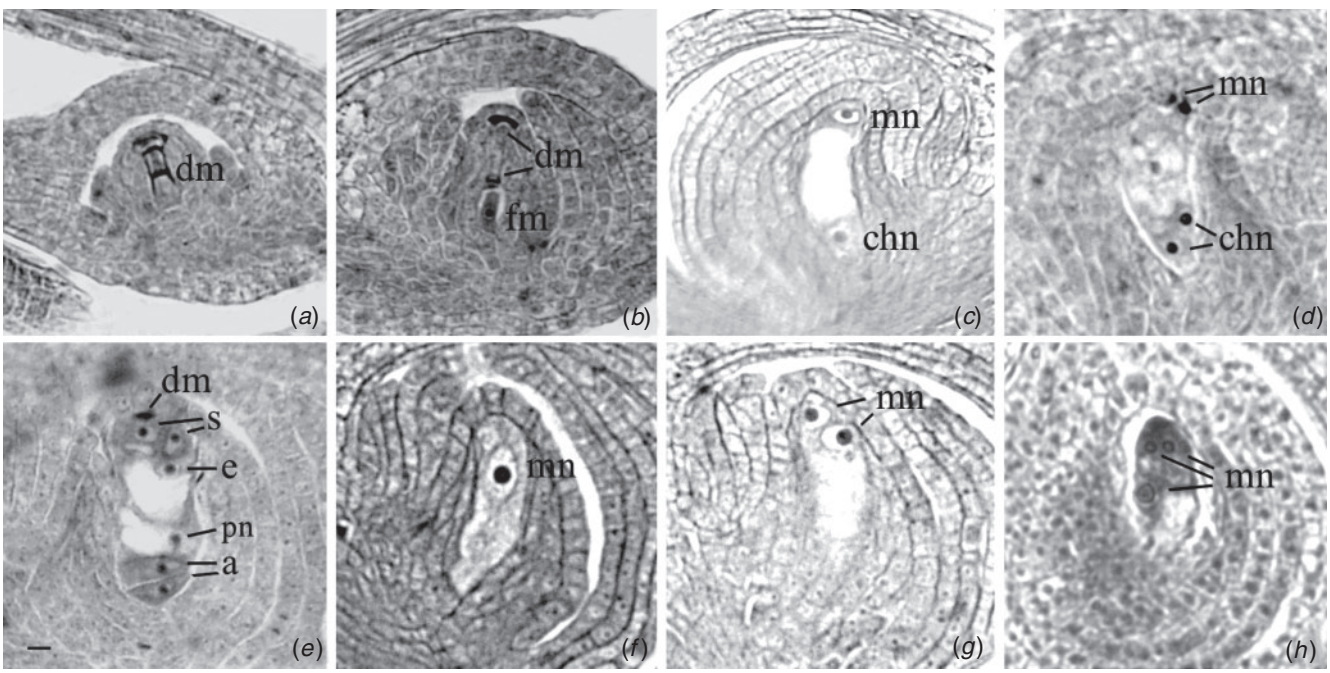

Fig. 2. Sexual (*a*–*e*) and diplosporous (*f*–*h*) embryo sac development in weeping lovegrass plants. Bar: 10 µm. Sections stained with safranine-fast green. (*a*) Three cells of the linear tetrad of megaspores. (*b*) Functional chalazal megaspore and degenerated megaspores. (*c*) Binucleated stage. (*d*) Tetranucleated stage. (*e*) Octanucleated stage. (*f*) Elongated megaspore mother cell. (*g*) Binucleated stage. (*h*) Tetranucleated stage. a: antipodal, chn: chalazal nucleus, dm: degenerated megaspore, e: egg, fm: functional megaspore, mn: micropilar nucleus, pn: polar nucleus, s: synergid.

and octanucleated stage (Fig. 2*e*) and proliferated antipodals were identified.

In spikelets collected from cultivars Tanganyika (Fig. 2), Don Juan, Don Eduardo, Don Pablo, Kromdraai, UNST1112 and UNST1131, EMMC (Fig. 2*f*) as well as binucleated and tetranucleated diplosporous embryo sacs (Fig. 2*g*, *h*) were observed. These observations clearly provided evidence for apomictic reproduction in these plant materials.

A total of 411 spikelets corresponding to genotypes UNST1122, UNST1131, UNST1112 and cultivars Tanganyika, Don Juan, Don Eduardo, Don Pablo, OTA-S and Kromdraai were analysed. Table 1 summarises these results, showing the number of pistils observed with aniline blue staining and discriminated by number of sexual pistils and apomictic pistils. Tetrads and degenerated megaspores indicated sexual events, while EMMC indicated apomictic events. The percentage of reduced and non-reduced embryo sacs was calculated for each genotype. Three fluorescent forms observed in UNST1131 and one in UNST1122 that were not clearly identified as corresponding to a sexual or apomictic process were considered to be aborted cells, and not included in the analysis.

The results obtained from sections stained with safranin-fast green are summarised in Table 2. Number of tetrads, degenerated megaspores and functional megaspore, and binucleated, tetranucleated and octanucleated embryo sacs were considered to be indicators of sexual reproduction. The EMMC, binucleated and tetranucleated embryo sacs in micropilar pole were deemed to be signs of apomictic reproduction. Percentages of sexual or apomictic events for each genotype were also calculated.

Progeny test

The progeny from cultivar Tanganyika displayed uniformity when analysed with all deployed primers, and showed the genotypic profile as the maternal plant, allowing classification as apomictic. The progeny of the diploid genotype $UNST1122_{R0}$ revealed polymorphisms between progeny plants and also with the maternal plant. The number of polymorphisms was dependent on individual primer, but all the progeny were distinct from the maternal plant, confirming 100% sexual reproductive mode in this material. Figure 3*a*, *b* show the RAPD profiles of typical progenies from apomictic and sexual plants, respectively. Progeny tests of genotypes UNST1131 and UNST1112 are shown in Fig. 3*c* and Tables 3 and 4. Based on number of polymorphisms, these genotypes display 9.0 and 7.0% sexual reproduction, respectively, and therefore were classified as facultative apomicts.

Flow cytometry

FCSS analysis of weeping lovegrass seeds derived from sexual and apomictic plants are shown in Fig. 4. Three main peaks were observed: a major peak corresponding to the embryo nuclei in G1, a second peak corresponding to the embryo nuclei in G2 and a third peak corresponding to endosperm nuclei in G1. The G1 stage represents the actual ploidy level of the sporophyte. At G2 stage chromosomes are duplicated before the cell division. The G2 peak was not present in endosperm material, because its mitotic activity is negligible.

The histogram of analysis based on a seed derived from the diploid genotype (Fig. 4*a*) showed three peaks corresponding to 2C (embryo G1), 3C (endosperm G1) and 4C (embryo G2) nuclei. As expected in a sexual plant, the embryo : endosperm DNA content ratio was 2 : 3 (2C : 3C). The histogram for a seed derived from the tetraploid cultivar Tanganyika ($2n = 4x = 40$) is shown in Fig. 4*b*. Peaks corresponding to nuclei contain 4C (embryo G1), 6C (endosperm G1) and 8C (embryo G2) were observed, although the ratio remained the same as in sexual plants. Peak position of independent samples with respect to *x*-axis was arbitrary and do not reflect the differences between ploidy levels.

Discussion

All analysed techniques, except from flow cytometry, were useful for phenotypic evaluation of reproductive mode. From use of cytological techniques, genotypes UNST1122 (2*x*) and OTA-S (4*x*) were classified as fully sexual, while Tanganyika (4*x*), Don Juan (8*x*) and Don Eduardo (6*x*) were considered to be full apomicts. Genotypes UNST1131 (4*x*), UNST1112 (4*x*) and Kromdraai (6*x*) were assigned to the facultative apomictic category.

Table 1. Aniline blue staining technique to distinguish apomictic versus sexual pistils in *Eragrostis curvula*

[a]Stages at which the reproductive mode can be assessed. PL: ploidy level; Sex: sexual pistils (tetrads + degenerated megaspores); Apo: apomictic pistils (elongated megaspore mother cells)

Genotype	PL	Number of pistils in optimum stage[a]	Number of pistils		Percentage of pistils	
			Sex	Apo	Sex	Apo
$UNST1122_{R0}$	2*x*	51	51	0	100	0
$UNST1122_{R1}$	2*x*	35	35	0	100	0
Tanganyika	4*x*	39	0	39	0	100
UNST1112	4*x*	13	2	11	15.4	84.6
UNST1131	4*x*	63	4	59	6.35	93.65
Don Eduardo	6*x*	17	0	17	0	100
Don Pablo	7*x*	3	0	3	0	100
Don Juan	8*x*	14	0	14	0	100
Total	–	235	92	143	–	–

Table 2. Pistils of *Eragrostis curvula* observed with the safranin-fast green staining technique to distinguish apomictic versus sexual plants

[a]Stages at which the reproductive mode can be assessed. PL: ploidy level; Sex: sexual pistils (dyads, tetrads, degenerated megaspores, functional megaspores, 2N, 4N and 8N embryo sac); Apo: apomictic pistils (elongated megaspore mother cells, 2N and 4N embryo sacs)

Genotype	PL	Number of pistils in optimum stage[a]	Number of pistils		Percentage of pistils	
			Sex	Apo	Sex	Apo
$UNST1122_{Ro}$	2*x*	95	95	0	100	0
$UNST1122_{R1}$	2*x*	19	19	0	100	0
OTA-S	4*x*	207	207	0	100	0
Tanganyika	4*x*	60	0	60	0	100
UNST1112	4*x*	49	7	42	14.3	85.7
UNST1131	4*x*	112	11	101	9.8	90.2
Kromdraai	6*x*	62	1	61	1.6	98.4
Total	–	604	340	264	–	–

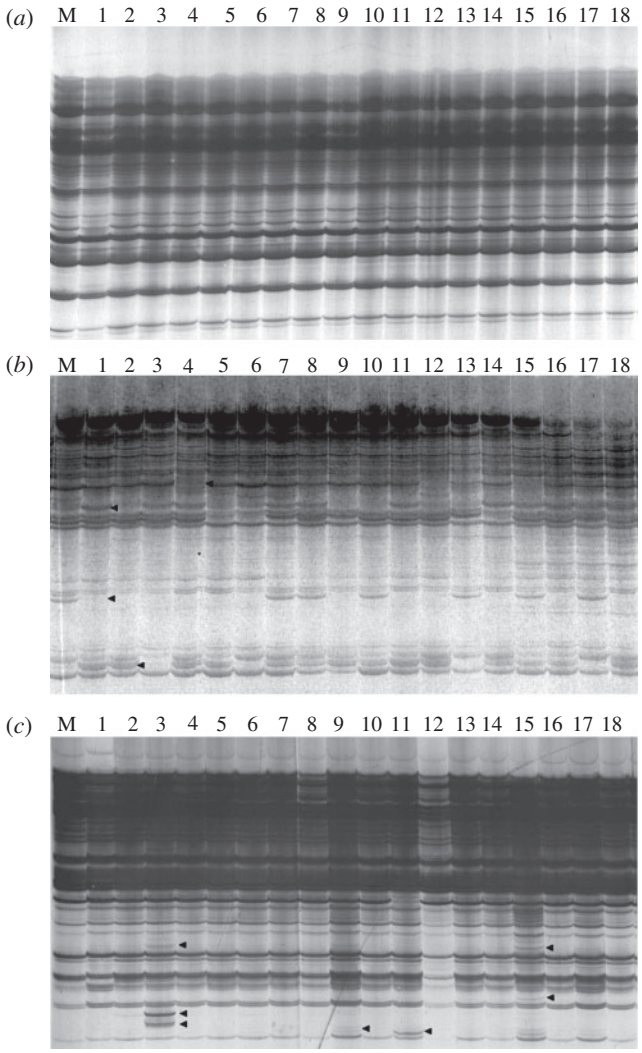

Fig. 3. Progeny test in three different materials of *Eragrostis curvula* evaluated with random amplified polymorphic DNA markers. (*a*) Apomictic cultivar (Tanganyika). (*b*) UNST1122. (*c*) UNST1131. M: maternal genotype. 1–18: individuals of the corresponding progeny. Arrows indicate polymorphic markers.

Table 3. Number of plants analysed and number and percentage of polymorphic individuals observed with random amplified polymorphic DNA markers in progeny tests

Mother plant	Number of plants	Number of polymorphic plants	Percentage of polymorphisms
UNST1131	99	9	9
UNST1112	15	1	7

Callose deposition observed in weeping lovegrass sexual plant UNST1122 follows the classical pattern previously described for sexual processes (Rodkiewicz 1970). From results shown in Table 1, it is also evident that callose is deposited on MMC and maintained through the early stages of embryo sac development in apomictic weeping lovegrass plants,

Table 4. Level of polymorphisms found in progeny plants of UNST1131 and UNST1112

Mother plant	Plant No.	Number of bands	Number of polymorphic bands	Percentage of polymorphisms
UNST1131	1	77	3	3.9
	2	77	3	3.9
	3	77	2	2.6
	4	77	3	3.9
	5	77	3	3.9
	6	77	2	2.6
	7	77	3	3.9
	8	77	2	2.6
	9	77	2	2.6
UNST1112	1	69	2	2.9

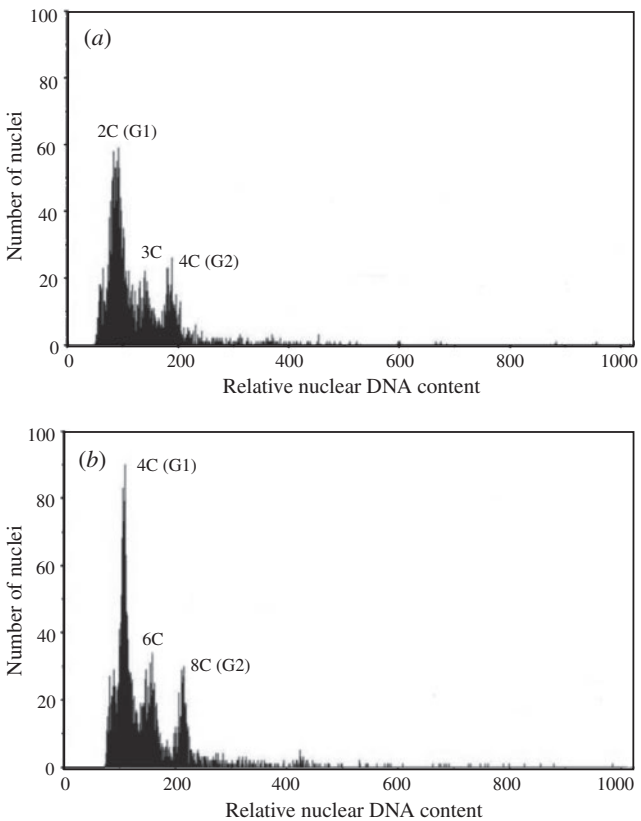

Fig. 4. Flow cytometry histograms of seeds originated from a sexual plant (UNST1122, $2n = 2x = 20$, panel *a*) an apomictic plant (Tanganyika, $2n = 4x = 40$, panel *b*). Panel (*a*): seeds originated from sexual diploid UNST1122 revealed nuclei at G1 originated from the embryo (2C peak), nuclei at G1 originated from the endosperm (3C peak), and nuclei at G2 originated from the embryo (4C peak). Panel (*b*): seeds originated from the apomictic tetraploid cultivar Tanganyika revealed nuclei at G1 originated from the embryo (4C peak), nuclei at G1 originated from the endosperm (6C peak), and nuclei at G2 originated from the embryo (8C peak). Peak position of independent samples with respect to *x*-axis is arbitrary and do not reflect the differences between ploidy levels.

contradicting previous reports for this and other diplosporous species. Absence of callose on MMC had been reported in the diplosporous species *Elymus rectisetus* (Carman *et al.* 1991), *Poa nemoralis* (Naumova *et al.* 1993), *Tripsacum* (Leblanc *et al.* 1995) and *E. curvula* (Peel *et al.* 1997). These authors suggested that other diplosporous MMC (*Antennaria*, *Taraxacum*, and *Ixeris* types) may also lack callose deposits on the MMC (Peel *et al.* 1997).

Peel *et al.* (1997) observed the absence of callose in MMC of 88 out of 89 weeping lovegrass pistils that were cleared stained in an aqueous aniline blue clearing medium. Callose restricted to the wall closest to the micropyle is occasionally observed in pistils of the diplosporous species *E. rectisetus* (Carman *et al.* 1991). These authors observed typical callose fluorescence in female meiocytes of sexual weeping lovegrass plants.

In a typical sexual process (*Polygonum* type), the cell wall of the megaspore and the megagametophyte cells increase in thickness throughout development. Medina *et al.* (1981) assigned a primary role to the process by which germinal tissue is isolated from vegetative tissues. The two most important changes in this process are the progressive thickening of the wall and the disappearance of the plasmodesms. Some researchers considered this physical barrier imposed by callose to be a prerequisite for the development of sexual processes. The lack of callose would allow access into MMC of nucellar signals, leading to triggering of mitotic instead of meiotic processes, which would explain the initial developmental stages of diplosporous apomixis (Peel *et al.* 1997). However, our data are not consistent with this hypothesis. Presence of a callose coverage was detected in appropriately staged pistils of both sexual and apomictic plants and in different cultivars and panicles.

Peel *et al*. (1997) also suggested that the precocious initiation of apomictic embryo sac should be accompanied by a precocious or altered activity of β-1,3-glucanase, which degrades callose from the functional megaspore before embryo sac formation. Although analysis of β-1,3-glucanase gene (*hpGluc*) expression in ovules of sexual and apomictic *Hieracium* plants suggests that the enzyme may play a role in callose degradation, no specific changes in temporal localisation of transcripts supporting this hypothesis could be observed (Tucker *et al.* 2001).

Even though callose deposition was observed on MMC from sexual and apomictic genotypes, these analyses allowed us to easily recognise the classic tetrads from the EMMC that are typical of diplospory, making this technique very attractive for characterisation of plants by their reproductive mode. Safranin-fast green staining technique confirmed the results obtained by callose deposition studies. This technique, although more laborious, was also more efficient, allowing identification of all the stages of embryo sac development proceeding from megasporogenesis to megagametogenesis, and also detection of low levels of sexuality in facultative apomict plants.

Results obtained with progeny tests were comparable to those obtained using cytoembryology, with 100% polymorphic progeny obtained from the sexual genotype UNST1122$_{R0}$, and 100% of uniform progeny obtained from the apomictic cultivar Tanganyika. For the facultative apomictic genotypes UNST1131 and UNST1112, the percentages were not identical (9.0 and 7.0% for progeny test versus 10.0 and 14.0% for cytology) since cytoembryological studies detect the ratio of reduced versus unreduced embryo sacs, and therefore the potential for apomixis, while progeny tests detect the real occurrence of offspring originating from apomixis or sexuality. Statistical analysis using Chi-square tests of homogeneity showed that the results are consistent and homogeneous across the techniques (progeny test and cytoembryological studies) in genotype UNST1131 (Chi-square = 0.63, $P = 0.729$) and genotype UNST1112 (Chi-square = 0.803, $P = 0.669$). The correlation between these results was determined by the capacity of all non-reduced/reduced embryo sacs to finally produce viable progeny. In a previous report (Cardone *et al.* 2006) based on progeny tests using molecular genetic markers, it was shown that genotypes UNST1131 and UNST1112 produced variable progeny, being classified as highly sexual (close to 100%). The reduction in the sexual reproduction level observed in the present study (after a 4-year period) may be explained by the genomic stress caused by *in vitro* culture and chromosome duplication using colchicine. These stressful situations could de-repress sexual reproduction, the apomictic level being subsequently restored. Other authors showed variation in reproductive mode after a period of tissue culture (Davies and Cohen 1992; Haluskova and Cellarova 1997; Polci 2000; Zappacosta 2009). Plant tissue culture has often regarded as a category of genomic stress, involving changes in DNA methylation and gene expression (Kaeppler and Phillips 1993). According to Voigt and Bashaw (1976), apomixis in *E. curvula* is controlled as a dominant character by a specific genomic region. It seems likely that epigenetic control permits expression of the sexual reproductive mode following application of genomic stress (Cervigni *et al.* 2008b).

There are few reported instances of full sexual tetraploids in apomictic species (Quarin *et al.* 2001; Acuña *et al.* 2009; Sartor *et al.* 2009; Quesenberry *et al.* 2010). Our results indicated that OTA-S genotype exhibited 100% sexual reproduction, according to cytoembryological analysis. This genotype apparently lacks the region that controls apomixes, or never expresses the trait. Performance of more detailed studies using OTA-S genotype is therefore of interest.

Our results also indicate that molecular markers are efficient for assessing the reproductive mode of *E. curvula* plants in order to differentiate between apomictic and sexual progenies in early stages of plant development. Similar results were obtained in other apomictic species, such as *P. notatum* (Ortiz *et al.* 1997) and *P. simplex* (Cáceres *et al.* 2001). RAPD markers also have a high discriminatory capacity, since a single primer can simultaneously amplify several different genomic regions, providing broad genome coverage (Williams *et al.* 1990). The present study reveals a strong level of concordance between cytological and molecular studies for determination of degree of apomixes, especially based on the behaviour of the facultative genotypes.

Flow cytometry analyses confirmed the outcomes of previous studies of apomictic development in weeping lovegrass (Voigt and Bashaw 1972, 1976) showing that apomictic embryo sacs have only one polar nuclei ($2n = 4x$), and that the endosperm is hexaploid ($3n = 6x$) as a product of pseudogamy. Therefore, comparison of embryo : endosperm DNA content ratio is not useful in this particular system to differentiate apomictic and sexual plants. The constancy of the 2 : 3 ratios in this forage species should be considered. Cereals, which are the most

important targets for transferring apomixis, are very sensitive to unbalanced embryo : endosperm ratio (Alleman and Doctor 2000). In other apomictic species, this ratio is altered because the embryo sacs have two non-reduced polar nucleui (Bicknell and Koltunow 2004), the endosperm being formed by the fusion of these nuclei ($4x+4x$) and a reduced male gamete ($2x$), rendering a $10x$ ($2n+2n+n=5n=10x$) endosperm. The one polar nucleus *Eragrostis* embryo sac type is very attractive as a model for transfer to cereal crops in the future, when the molecular pathways underlying apomixis have been clearly identified as to allow the control of the trait in major crops. Cereals are very sensitive to changes at this level.

Of the four techniques evaluated in this study (callose deposition, classical cytoembryology with safranin-fast green, progeny tests and ploidy analysis), progeny tests and cytoembryological studies (callose and classical) were shown to be most suitable for analysis of the reproductive mode, allowing characterisation of obligate apomicts, facultative apomicts and sexual plants with a minimal chance of error. A combination of progeny tests using molecular genetic markers and callose tests can simplify and accelerate phenotypic evaluation for this trait in weeping lovegrass mapping populations. Incorporation of the callose test reduces the time needed for preparation and observation of materials as only the major criteria for discrimination between sexual and apomictic pistils are displayed. However, this technique does not allow observation of the tendency of megagametogenesis to produce a lower number of embryo sacs for each spikelet. In this sense, the best approach to corroborate the analysis and determine reproductive mode is the safranin-fast green staining technique. The presence of callose in MMC that originate from apomictic plant megasporogenesis calls into question previous observations and merits further study.

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