Acta Bot. Neerl. 42(3), September 1993, p. 299–312

Quantitative analysis of aposporous parthenogenesis in *Poa pratensis* genotypes

T. NAUMOVA, A. P. M. DEN NIJS* and M. T. M. WILLEMSE‡

*Komarov Botanical Institute, St Petersburg, Russia and *Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen, The Netherlands, and ‡Department of Plant Cytology and Morphology, Agricultural University Wageningen, The Netherlands*

SUMMARY

Several embryological and cytological techniques were developed to determine the degree of aposporous parthenogenesis vs. sexuality in different genotypes of the facultative apomictic grass *Poa pratensis* L. Fixed embryo sacs were mechanically isolated and individually examined with phase-contrast and interference-contrast light microscopes. The degree of apospory was calculated as the ratio of embryo sacs with an embryo but without endosperm and the total number of embryo sacs studied per plant. The degree of apospory varied between 9 and 76% in nine plants. Photocytometry and flow cytometry were used to determine ploidy levels in embryo and endosperm of individual embryo sacs in order to determine their origin, asposporous vs. sexual. Results based on the calculated degree of apospory agreed with the degree of apomixis deduced from homogeneity scores in field progeny tests. Callose deposition on the megaspore mother cell during megasporogenesis was studied in the same set of genotypes and the percentage of abnormal, micropylar callose deposition closely agreed with results both of the embryo sac analysis and the progeny tests. Combined use of these techniques yields quantitative data on the aposporous tendency of individual genotypes of *Poa pratensis*.

Key-words: apomixis, endosperm, callose deposition, embryo sac, cytophotometry, flow cytometry, *Poa pratensis*, *Poa* spp.

INTRODUCTION

Poa pratensis is a cosmopolitan species with a high variation in chromosome number. A tendency to apomixis is one of the characteristic features of this species. Most genotypes are facultative, i.e. they contain both apomictic and sexual embryo sacs. The main type of apomixis in this species is apospory followed by parthenogenesis. However, apomictic seed formation depends in *P. pratensis* as in many other apomictic species, on endosperm development as a result of a single fertilization. Apospory and diplospory usually involve two major components: formation of unreduced (diploid) embryo sacs and development of the embryo by parthenogenesis or apogamety. A binary terminology proposed earlier (Naumova 1990) to define these phenomena is used in this paper. For example, aposporous parthenogenesis means asexual embryo development from the diploid egg cell of an aposporous embryo sac.

Some characteristic features for the identification of apomixis in *Poa* were suggested from embryological investigations by Mirochnichenko (1964). Precocious embryo development before pollination and endosperm formation were stated as a characteristic feature of parthenogenesis for *P. pratensis* and some other species. It was suggested that parthenogenesis is in close relation to apospory, but no ploidy levels for endosperm and embryo were determined.

A quick and reliable method to estimate the balance between sexuality and apomixis in natural populations as well as in progeny from crossings would be extremely useful for both plant breeding and ecological studies.

Classical cytological techniques are useful neither for investigation of numerous ovules and embryo sacs nor for estimation of ploidy levels. To determine the kind of reproduction (sexual or apomictic) as well as the degree of apomixis in *P. pratensis*, several techniques should be applied on the same genotypes. Interest in the possibilities for manipulating apomixis has recently increased for breeding as well as for some genetic engineering programmes. Therefore, it has become obvious that time-saving and informative techniques need to be developed to screen for apomixis among wild and cultivated species as well as to determine the degree of apomixis and to estimate endosperm and embryo ploidy levels.

There are several recent developments in the techniques used for megasporocyte and total embryo sac analysis. Firstly, clearing, staining and squash methods are being improved (Herr 1971; Abeln *et al.* 1984; Crane and Carman 1987; Jongedijk 1987a; Kojima *et al.* 1991; Kojima & Nagato 1992). These techniques are very useful for the investigation of sporo- and gametogenesis, but they are less suited to the study of embryo sacs after pollination. The clearing squash technique was not suitable for our investigation of aposporous embryo sacs and parthenogenesis in *P. pratensis* because of the strong integumental barrier and the thickness of the ovule.

Secondly, progress has been made in the enzymatic degradation of the cell walls of ovules to isolate megasporocytes, embryo sacs and developing embryos (Enaleeva *et al.* 1972; Solntseva & Levkovskij 1978; Hu *et al.* 1985; Jongedijk 1987b; Van der Maas *et al.* 1993; Wagner *et al.* 1988; Van Went & Kwee 1990). This technique makes it possible to obtain viable embryo sacs or embryos for experiments or analysis. (For a recent review of the isolation of male and female gametes in higher plants see Theunis *et al.* 1991.)

The third aspect of technique improvement involves the modification of existing methods to dissect complete embryo sacs from the ovule without maceration. This technique has been developed by various authors in Russia (Poddubnaja-Arnoldi 1954; Petrova 1970; Orlova & Avalkina 1985; Oryol 1986) and enables the investigation of numerous embryo sacs, identification of polyembryony, cytological analysis of the endosperm and estimation of embryo sac fertility. This technique was modified in the authors' investigations (Naumova *et al.* 1992).

Finally, an approach based on the callose deposition in ovules of sexual and apomictic species is being developed. Callose walls in microsporogenesis were originally described more than 100 years ago. Investigations on callose deposition in megasporogenesis started before the seventies (Rodkiewicz 1967). Callose was found partly surrounding the metasporocyte and tetrads in 43 species from 14 families of angiosperms with the monosporic type of embryo sacs, but it was not found in the case of tetrasporic embryo sacs (Rodkiewicz 1970). A first investigation of callose events in the case of apomixis was undertaken by Carman *et al.* (1991). These authors showed by light microscopy, differences in thickness of the megasporocyte cell wall between

diplosporic *Elymus rectisetus* and sexual *E. scabrus*. Absence of callose deposition around the megasporocyte in the case of diplospory was associated with a thinner cell wall. This approach of identifying the fate of the megasporocyte by studying callose deposition is developed for *Poa* in the present investigation. The aim of this study is to determine via different qualitative and quantitative techniques, the degree of apomixis of specific *P. pratensis* genotypes in order to better choose crossing parents for a breeding programme.

MATERIAL AND METHODS

Twelve *P. pratensis* genotypes under study were derived from cultivars Cynthia, Barblue, Asset and Nimbus by selecting offtype twin seedlings (Den Nijs & Winkelhorst 1992), included in a CPRO-DLO research programme, Wageningen, The Netherlands. The genotypes showed a different tendency towards apomixis as estimated from the degree of homogeneity of the offspring. Inflorescences were collected from greenhouse grown plants, 2–11 days after self- or open-pollination, fixed in formaldehyde–alcohol–acetic acid (FAA) and stored in ethanol 70°. *P. annua*, sexual, and *P. nemoralis*, diplosporous, were collected in natural populations in Wageningen, The Netherlands. A part of the material was investigated with classical cytological techniques. Flowers were embedded in paraplast, sectioned and stained with safranine/aniline blue and investigated with a light microscope.

For the analysis of complete, dissected embryo sacs, the ovules were dissected from the ovaries, and embryo sacs were taken out from the ovules by hand using fine needles under a binocular at 40 × magnification. The embryo sacs were stained with the DNA-specific fluorochrome DAPI (10^{-5} g DAPI buffered, pH=4·0) solution for 10 min and embedded in glycerine–gelatine. Isolated embryo sacs were examined and photographed under phase-contrast and interference-contrast microscopes.

For the measurement of DNA-amounts a cytophotometer was used composed of a Zeiss-junior microscope equipped with a Ploem incident UV light with an excitation wavelength of 365 nm from a high pressure 100-W mercury lamp. A motor-driven Scott interference S20 filter for the emission spectrum was used and an RCA 31034 photomultiplier with fluke 412B high-voltage power-supply detected the signal. A Groerts RE541 recorder registered the signals in millivolts. Using 900 V power the maximum intensity was determined at about 470 nm wavelength in millivolts. Using a 6 × ocular and 40 × objective lens, measurements were taken from at least 10 different areas of $16 \times 16\ \mu m^2$. The amount of DNA was measured in intact nuclei of the endosperm cells, the embryo and the sporophytic nucellus cells. The 16×16-μm^2 area used just covers a single endosperm nucleus. In the case of the much smaller nucellus nuclei, the measured value was doubled. In the case of dividing endosperm, commonly two classes of intensity were measured, one from G1 and the other from S or G2 phases. The data of the interphase class were used in the comparison. Factors influencing variation in measurements are both technical: lamp condition, adjustment of energy input between different measurements; and preparative: thickness of object, dimensions and shape of the nuclei, presence of starch.

For determination of the nuclear DNA content by flow cytometry, fresh post-pollination ovaries were carefully chopped with a sharp razor-blade to release the nuclei from the plant tissue. The nuclei were transferred to nuclear-isolation buffer with pH=5·8, containing 0·25% of a 1 mg litre^{-1} DAPI solution (Verhoeven *et al.* 1990).

Table 1. Characteristics of aposporous parthenogenesis in *Poa pratensis* genotypes

Cultivar	Plant no.	Investigated number of embryo sacs	Class 1 +endosperm +embryo	Class 2 −endosperm +embryo	Class 3 −endosperm −embryo	Minimum degree of apomixis* (%)
Cynthia	143	59	7	23	29	76
Barblue	47	42	5	4	33	44
Asset	64	66	9	12	45	58
	84	35	2	2	31	50
	87	42	2	6	34	75
	52	>50	0	0	>50	sterile
Nimbus	114	104	47	27	30	36
	121	64	49	5	10	9
	90	>50	0	0	>50	sterile

*Ratio of the number of embryo sacs without endosperm, but with embryo (class 2) over the total number of analysed embryo sacs, excluding the sterile ones (class 3).

After filtration through a 50 μm nylon gauze filter, samples were analysed on a Partec PAS-II flow cytometer (Partec, Munster, FRG) with a UG5 excitation filter, TK 520 dichroic mirrors, and a GG435 long-pass filter. Channel analysis was performed with the standard software of the PAS-II (Verhoeven *et al.* 1990).

Callose deposition was studied in eight aposporous and sexual *P. pratensis* genotypes, in sexual plants of *P. annua* and in plants of diplosporous *P. nemoralis*. Fresh ovaries were stained on a microscope slide with a 0·005% solution of aniline blue in 0·15 M $K_2H_2PO_4$, embedded in glycerine and covered by a cover glass. Ovules were released from the ovaries by gentle pressure and examined with 420 nm incident light using a UV microscope. Photographs were taken on Kodak technical pan film TP135.

RESULTS

Characteristics of seed formation

Analysis of complete ovules and dissected embryo sacs, 2–7 days after pollination, revealed different possibilities for development between the genotypes. The examined embryo sacs could be classified into three groups with respect to the development of embryo and endosperm: (1) embryo sacs containing both embryo and endosperm; (2) presence of an embryo, but no endosperm; (3) neither an embryo nor endosperm present. The data on open- and self-pollination were quite similar and could be combined. These results are presented in Table 1.

Embryo sacs with embryo and endosperm (class 1) will develop and produce viable seeds. A determination of the ploidy level of the endosperm of the seeds can determine whether the seed is of aposporic or sexual origin.

Embryo sac formation with an embryo and without endosperm (class 2) is rather common in these genotypes of *P. pratensis* (Table 1). These embryos can only result from parthenogenesis of the egg cell. The absence of endosperm around an embryo and the presence of intact synergids (Fig. 1a) showed that no fertilization occurred. These embryo sacs degenerated and no seed set occurred so there is no autonomous endosperm development. The structure of the embryos developing without endosperm

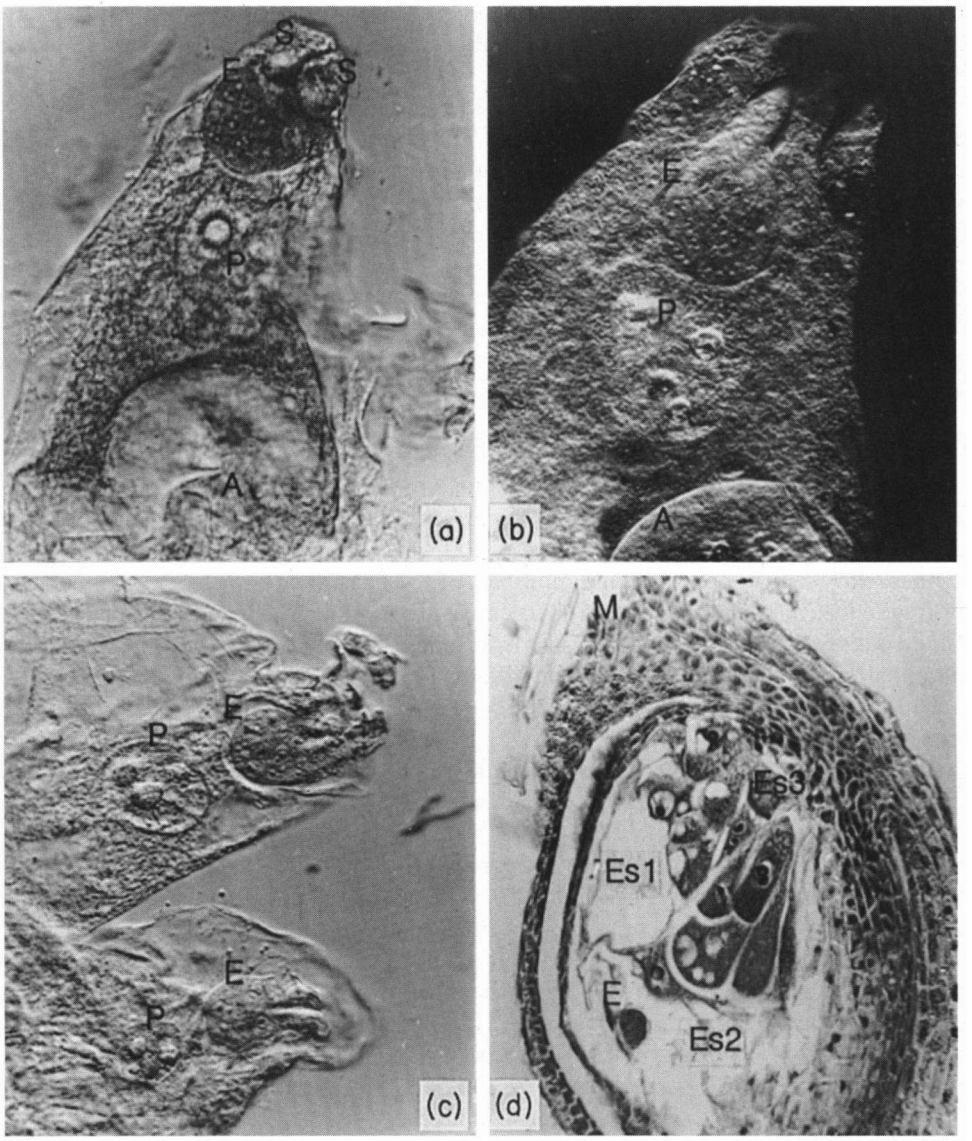

Fig. 1. Apospory and parthenogenesis in *P. pratensis* genotypes. (a) Isolated embryo sac with parthenogenetic embryo (E), intact synergids (S), two polar nuclei (P), antipodals (A) located in the chalazal part of the embryo sac (400 ×). (b) Isolated embryo sac with parthenogenetic globular embryo (E) (advanced stage), without endosperm formation, polar nuclei were in close contact, antipodals were present in chalazal part of the embryo sac (phase-contrast) (400 ×). (c) Two isolated embryo sacs from one ovule; each of them had an embryo, polar nuclei (no endosperm) and antipodals (phase-contrast) (400 ×). (d) Ovule with three embryo sacs in different position (section from paraplast embedded material, stained by safranin–aniline blue); one embryo sac (ES1) with visible egg apparatus close to the micropyle, a second one (ES2) with developing (E) embryo had a lateral position; a third embryo (ES3) sac partly visible between ES1 and ES2 (M-micropyle) (200 ×).

was globular with a distinct suspensor and normal cell pattern (Fig. 1b). More advanced stages of embryo development without endosperm were not found *in vivo*. The observed embryo sacs without an embryo and endosperm were sterile (class 3). The degree of

sterility in the genotypes under study was high. Two plants (no. 52 and 90) differed from the others, producing only sterile embryo sacs, so they were classified as sterile (Table 1).

One ovule usually contains one embryo sac, but in a minority of the ovules two or more embryo sacs were detected (Fig. 1c,d). The developmental capacity of two embryo sacs in one ovule differed. Some of them possessed only an embryo but no endosperm (see Fig. 1c), whereas others had both an embryo and endosperm, likely to succeed in viable seeds, and some contained neither embryo nor endosperm resulting in sterile seeds.

Based on the observed number of embryo sacs without endosperm, a minimum percentage of apomixis is calculated as the ratio of the number of embryo sacs with an embryo and without endosperm (class 2) and the total number of investigated embryo sacs excluding the sterile ones (Table 1).

Endosperm and embryo DNA amounts and ploidy levels

The cytophotometric data on the embryo and endosperm ploidy level are presented in Table 2. The value for the nucellus DNA amount is taken as $2n$ and the ploidy levels of endosperm and embryo are deduced from this value. It is realized, that chromosome numbers in *P. pratensis* are usually multiples of the basic number $x=7$, and aneuploids are frequent. Endosperm ploidy level was measured in five genotypes. About one-third of the endosperms were $5n$ and two-thirds were $3n$. Three plants of the cultivar Barblue contained both $5n$ and $3n$ endosperms. Plant 143 contained $5n$ endosperm. Eight $3n$ endosperms and a single case of probably $2n$ endosperm were observed in plant 121. One investigated embryo sac of plant 150 with $5n$ endosperm contained two $2n$ embryos. Two embryo sacs developing in one ovule were found in plant 143, one with $5n$ endosperm and a $2n$ embryo while the other contained only a $2n$ embryo.

The nuclear DNA content was also measured by flow cytometry in the ovaries and leaves of two plants (Fig. 2). For technical reasons slight shifts of peak positions of the ovaries and leaves were observed. Plant 143 showed a $2n$ level for somatic tissue and an accompanying $4n$ peak ascribed to cells in the S-phase of this fast developing structure. Besides this, a small $5n$ peak is visible, ascribed to the endosperm (Fig. 2a,b). Naturally the small number of endosperm nuclei in the measured sample resulted in a small peak relative to the $2n$ peak. Ovaries of plant 121 show a $2n$ and a $4n$ peak for the somatic ovary nuclei and two peaks for the endosperm, i.e. $3n$ and $5n$ (Fig. 2c,d). In the leaf samples only the $2n$ peaks are present.

Callose deposition during megasporogenesis of some species of Poa

Two different patterns of callose deposition during megasporogenesis in *P. pratensis* genotypes were distinguished: (1) that in which callose was observed around the megasporocyte and tetrad (Figs 3a and b); (2) that in which callose was found as a micropylar cap only—this was estimated as an abnormality which may indicate an apomictic development pattern (Figs 3c and d). Both types of callose deposition around the megasporocyte or tetrad were observed in different ovules of the same plant. In some ovules autofluorescence was present in the region of the hypostase as well. An analysis of the callose patterns in different *Poa* species is given in Table 3. The degree of apomixis was calculated as the ratio of the number of ovules with only micropylar callose deposition and the total number of investigated ovules. This degree of apomixis is different between genotypes. Plant 143 showed the highest degree of abnormality

Table 2. Endosperm and embryo DNA amount and estimated ploidy levels of *Poa pratensis* genotypes

Cultivar	Plant no.	Estimated chromosome number	Number of endosperms investigated	Endosperm DNA amount	Estimated ploidy level of endosperm	Embryo DNA amount	Ploidy level of embryo	Nucellus DNA amount (2*n*)
Barblue	47	82	2	1·9 ± 0·6	3*n*			1·6 ± 0·2
			5	3·8 ± 0·7	5*n*			
Barblue	51	54	5	2·0 ± 0·3	3*n*			1·2 ± 0·2
			3	3·7 ± 0·6	5*n*			
Barblue	150	76, 108	4	2·6 ± 0·3	3*n*			1·8 ± 0·6
			2*	4·3 ± 0·3	5*n*	1·4 ± 0·4	~2*n*	
						1·2 ± 0·4	~2*n*	
Cynthia	143	54, 76	1‡	4·5 ± 0·5	5*n*	1·2 ± 0·4	~2*n*	1·8 ± 0·4
						1·3 ± 0·6	~2*n*	
Nimbus	121	77, 82, 102	8	2·9 ± 0·5	3*n*			1·8 ± 0·4
			1	1·6 ± 0·4		~2*n*		

*Two embryos in one embryo sac.
‡Two embryo sacs in one ovule.

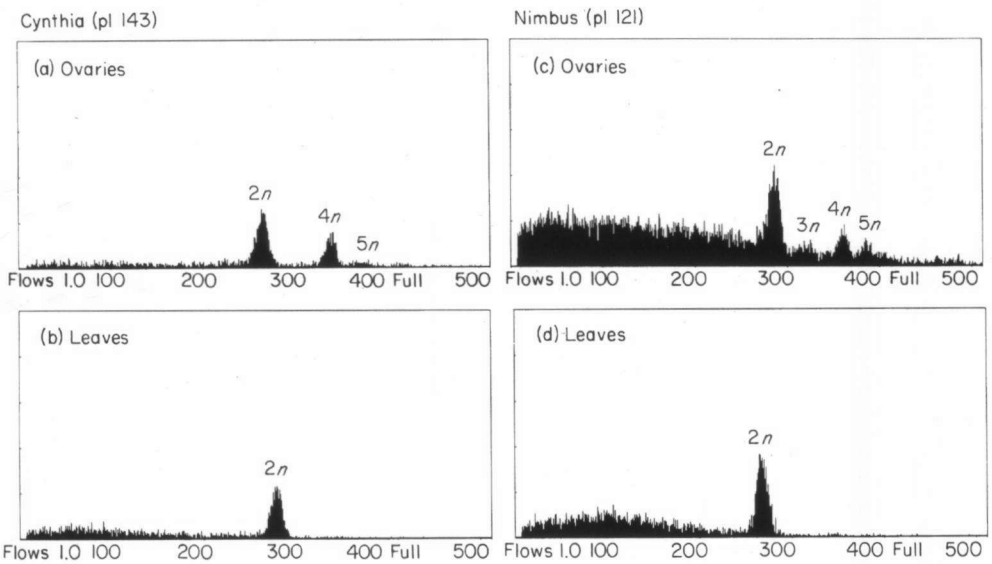

Fig. 2. Nuclear DNA content in ovaries and leaves of *P. pratensis* genotypes determined by flow cytometry Cynthia (plant no. 143), ovaries (a) and leaves (b). Nimbus (plant no. 121), ovaries (c) and leaves (d).

whereas plant 121 had the lowest percentage of ovules with micropylar callose deposition.

In *P. annua* no ovules with micropylar callose deposition were observed, five megasporocytes and seventeen tetrads showed normal callose deposition.

More than 50 ovules of *P. nemoralis* showed no sign of callose at all. This phenomenon occurred in ovules of different sizes from the very young to those developed through meiosis.

DISCUSSION

The present report combined three techniques for the quantitative analysis of apomixis in *Poa*: dissected embryo sac analysis, ploidy estimates by cytophotometry and flow cytometry, and determination of callose deposition in apomictic *P. pratensis* genotypes with aposporous parthenogenesis, in *P. nemoralis* with diplospory and in sexual *P. annua*. Embryological investigations on diplospory in *P. nemoralis* and the sexual process in *P. annua* were previously described (Mirochnichenko 1978; Khristov & Terziisky 1979). A critical evaluation of the usefulness of these techniques for the determination of the degree of apomixis in individual genotypes is made in the present study.

The complete embryo sac dissection method appears to be suited to rather large-scale studies. This method requires a certain amount of skill, but it enables one to observe a large number of complete embryo sacs after anthesis. It is concluded that most of the *P. pratensis* genotypes show a tendency to apomictic seed development, but there are differences between genotypes in the degree of apomixis and sexuality. Some geno-types show predominantly apomictic seed development, while in others apomictic and amphimictic potentialities are more or less in balance. For one genotype, plant121, sexual processes predominate. Two plants appear to be sterile. These both originated

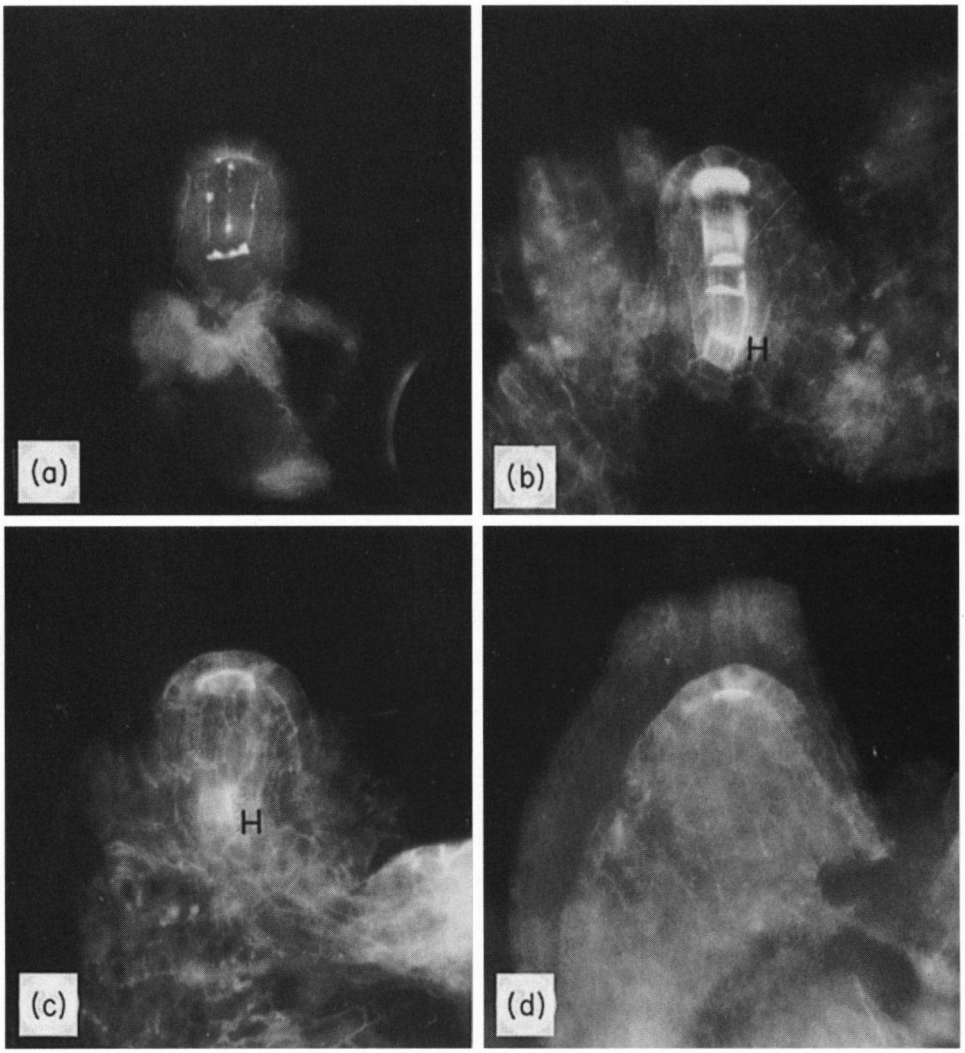

Fig. 3. Callose deposition during megasporogenesis. (a) Callose deposition in megasporocyte cell wall (300 ×). (b) Callose deposition in tetrad cell walls (H=hypostasis) (300 ×). (c) Micropylar cap of callose above the megasporocyte (300 ×). (d) Disappearance of callose cap at some later stage of ovule development (300 ×).

from a seed with two seedlings (twin seedlings) and were weak. Comparison of their chromosome number with that of the parent variety by flow cytometry revealed that both have half the chromosome number of the parent, and that they were most probably parthenogenetic haploids. These haploids are known to occur infrequently in twin seedlings in *P. pratensis* (Den Nijs 1990; Den Nijs & Winkelhorst 1992). Because of the low number of plants investigated, it was impossible to fully characterize all varieties for their degree of apomixis. The degree of sterility in the genotypes under study was probably high because greenhouse conditions affect pollen dispersal and fertilization. Embryo maturation and seed formation without endosperm were never observed.

Table 3. Normal and micropylar callose deposition in ovules of *Poa pratensis* genotypes and *P. annua*

Species/ cultivar	Plant no.	Number of ovules investigated	Normal callose deposition		Micropylar callose deposition only	Apomixis* (%)	Minimum degree of apomixis from Table 1 (%)
P. pratensis			Megasporocytes	Tetrads			
Cynthia	143	22	4	—	18	82	76
Barblue	47	32	12	6	14	44	44
Asset	64	44	13	7	24	55	58
	84	21	9	—	12	57	50
	87	25	13	4	8	32	75
Nimbus	114	42	27	3	12	29	36
	121	74	33	27	14	19	9
	90	16	7	2	7	44	sterile
P. annua		22	5	17	0	0	sexual

*Calculated as the ratio of ovules with micropylar callose deposition over the total number of investigated ovules.

The determination of the proportion of apomixis is based on the sum of the developing seeds with an embryo and with endosperm, and the non-developing, endospermless seeds. Only after estimation of the fraction of apomictic seeds in the category of seeds with an embryo and with endosperm ($5n$), may the results be correlated with the breeders' estimations based on the percentage of aberrant plants in an offspring field test. Cytometry and flow cytometry can be used for this approach. In recent years flow cytometry has been used more intensively in biology (Verhoeven *et al.* 1990; Ulrich & Ulrich 1991).

Estimation of the ploidy level of the endosperm is important in order to determine the origin of the embryo sac as well as the presence or absence of fertilization. Knowledge about endosperm development and its ploidy level in apomictic plants is scanty (Nogler 1984; Czapik 1991). Pentaploid ($5n$) endosperm ($2n+2n+n$) is an indiction of aposporic embryo sac formation and endosperm development as a result of fertilization. This phenomenon was observed in all of the genotypes under study. Triploid ($3n$) endosperm indicates a reduced haploid embryo sac formation and endosperm development after fertilization ($n+n+n$). Polyploidization in endosperm nuclei was not found at this stage.

The data show that endosperms of reduced as well as unreduced embryo sacs start to develop only after fertilization. The presence of both $3n$ and $5n$ endosperm in different ovules of one plant shows a tendency to normal (n) and aposporous ($2n$) embryo sac formation in the same plant, i.e. apomictic and amphimictic seed formation coexisting in the same plant. This phenomenon was observed practically in all investigated *P. pratensis* genotypes. Similar data exist for other *P. pratensis* genotypes (Abeln *et al.* 1984) and other angiosperms (Naumova 1992).

The results of the cytological analysis of complete embryo sacs and of the cytophotometry usually agreed for those plants for which both data were obtained.

It is still questionable whether endosperm development without fertilization occurs at all in *P. pratensis*. Estimations of nuclear DNA amounts showed that viable seeds in *P. pratensis* only result from amphimixis after double fertilization or from parthenogenetic embryo development with endosperm formation after single fertilization. The single $2n$ endosperm in plant 121 is thought to be quite exceptional.

Flow cytometry was used to evaluate the possibilities of this technique for the investigation of plant reproductive structures. The results of flow cytometry of the ovaries of plants 143 and 90 (data not shown) closely agreed with the cytophotometry results and the cytological analysis (Table 1). However, plant 121 according to flow cytometry results shows development of $3n$ and $5n$ endosperm (Fig. 2c), in contrast to the cytophotometry indicating $3n$ endosperm only (Table 2). Cytological study showed the lowest degree of apomixis for that plant. It is concluded that flow cytometry can be effectively used in plant embryology because a high number of nuclei can be analysed from small samples such as individual excised ovaries.

Callose deposition in ovules of aposporous plants is reported here for the first time. The degree of aposporous parthenogenesis in *P. pratensis* genotypes estimated by embryo sac analysis should be compared with the degree of abnormal callose deposition in megasporogenesis (Table 3). It appears that the estimated tendencies to apomixis and sexuality in the plants under study by these methods are comparable in spite of the fact that callose is already present at an early stage of the development, before fertilization. This similarity can be partly explained by early embryological investigations (Kiellander 1941) which showed that in aposporous *P. pratensis* plants megasporocyte meiosis may be normal or abnormal. Abnormal deposition of the callose in aposporous *Poa* is an

indication of such an abnormality in megasporocyte development. Recently Carman *et al.* (1991) demonstrated by light microscope investigations, distinct differences in the thickness of the megasporocyte cell wall between sexual *Elymus scabrus* and diplosporous *E. rectisetus*. No callose deposition in and around the megasporocyte cell wall was observed in diplosporous *E. rectisetus*, while the sexual species contained normal callose deposition in and around the megasporocyte. However, the significance of the differences in callose deposition during megasporogenesis of species with normal sexual reproduction (*P. annua, E. scabrus* and others) and species with apomixis, e.g. apospory (*P. pratensis*) or diplospory (*P. nemoralis, E. rectisetus*) needs to be identified by further investigations.

Classical cytological serial section techniques are unable to distinguish beyond doubt the individual embryo sacs in *P. pratensis* as in other grasses. As an illustration Fig. 1d may be used. It shows an ovule with three embryo sacs (only one slide from a serial). The position of these embryo sacs in the ovule is different, because the egg apparatus of the first and the third embryo sacs and the developing embryo of the second embryo sac have opposite orientations. From such a position the embryo developing from the egg apparatus cell might be incorrectly identified as a nucellar embryo. However, the data about nucellar embryony in grasses are inconclusive.

Besides the difficulties in classification, the amount of time necessary to obtain the data is prohibitive for large-scale experimentation. Therefore, alternative methods for quantification of apomixis are desirable. The progeny test which deduces the degree of apomixis from the homogeneity of the offspring of individual plants, can only give an approximate evaluation (Den Nijs & Van Dijk 1992). As an alternative Abeln *et al.* (1984) attempted to predict the reproductive character of *P. pratensis* plants by study of the early stages of ovule and embryo sac development. Recently, an auxin treatment for estimation of the degree of apomixis was proposed (Matzk 1991). Examination of morphological characteristics of the induced developing structures remains necessary.

The isolated embryo sac analysis proposed in this study could possibly be improved by making use of digestive enzymes to isolate embryo sacs (Krantz *et al.* 1991; Van der Maas *et al.* 1993). This method could, after optimization, yield large numbers of embryo sacs for study.

The different methods used in the present study each give an estimate of the level of apomixis. The comparison of the results reinforces the conclusion because each technique has its own merits and resolution. Mechanical embryo sac isolation reveals the type of apomixis and other abnormalities. The cytophotometry and flow cytometry show ploidy levels as an individual characteristic and at population level, respectively. The callose test may predict apomixis, including the type of apomixis. All tests as applied to *P. pratensis* reveal the great variability within this species, and further refinement, especially of the callose deposition method may yield a fast, quantitative estimation method.

ACKNOWLEDGEMENTS

The authors are indebted to G.D. Winkelhorst for care of the plants, S. Massal for the photographs, A.B. Haasdijk for the illustrations, and M. Wagenvoort for critical reading of the manuscript. One of the authors (T.N.) was supported by a grant from IAC, Wageningen, The Netherlands.

REFERENCES

Abeln, J.S., Wilms, H.J. & Van Wijk, A.J.P. (1984): Initiation of asexual seed production in Kentucky bluegrass, *Poa pratensis* L. In: *Proceedings of the 8th International Symposium on Sexual Reproduction in Seed Plants, Ferns and Mosses.* 160–164. Wageningen, The Netherlands.

Carman, J.G., Crane, C.F. & Riera-Lizarazu, O. (1991): Comparative histology of cell walls during meiotic and apomictic megasporogenesis in two hexaploid Australian *Elymus* species. *Crop Sci.* **31:** 1527–1532.

Crane, C.F. & Carman, J.G. (1987): Mechanisms of apomixis in *Elymus rectisetus* from eastern Australia and New Zealand. *Am. J. Bot.* **74:** 477–496.

Czapik, R. (1991): Some research problems of the endosperm in angiospermae. *Pol. Bot. Stud.* **2:** 109–120.

Den Nijs, A.P.M. (1990): Experimenting with apomixis and sexuality in *Poa pratensis. Apomixis Newslett.* **2:** 52–54.

Den Nijs, A.P.M. & van Dijk, G.A. (1992): Apomoxis and plant breeding. In: Hayward, M.D., Bosemark, N.O. & Romagosa I. (eds): *Plant Breeding Principles and Prospects.* Chapman & Hall, London.

Den Nijs, A.P.M. & Winkelhorst, G.E. (1992): Apomixis: a mixed blessing to the breeder of polyploid *Poa pratensis.* In: Veronesi, F., Bullitta, S. and Caredda, S. (eds): *Ploidy and Chromosome Manipulation in Forage Breeding*, 45–52. (*Proceedings of the 17th Eucarpia Fodder Crops Section Meeting*, Alghero, It.).

Enaleeva, N.Kh., Turnov, V.S. & Khokhlov, S.S. (1972): Dissection of the embryo sacs of some angiosperms by means of tissue maceration. *Cytol. Genet.* **6:** 439–441.

Herr, J.M., Jr. (1971): A new clearing-squash technique for the study of ovule development in angiosperms. *Am. J. Bot.* **58:** 785–790.

Hu, S.Y., Li, L.G. & Zhu, C. (1985): Isolation of viable embryo sacs and their protoplasts of *Nicotiana tabacum. Acta. bot. sin.* **27:** 337–344 (English summary).

Jongedijk, E. (1987a): A rapid methyl salicylate clearing technique for routine phase-contrast observations on female meiosis in *Solanum. J. Microsc.* **146:** 157–162.

Jongedijk, E. (1987b): A quick enzyme squash technique for detailed studies on female meiosis in *Solanum. Stain Technol.* **62:** 135–142.

Kiellander, C.Z. (1941): Studies on apospory in *Poa pratensis* L. *Svensk. bot. Tidskr.* **35:** 321–332.

Khristov, M.A. & Terziisky (1979): Origin of twin-plants in some apomictic species of genus *Poa* L. *Phytologia, Bulg. Acad. Sci.* **11:** 3–14.

Kojima, A. & Nagato, J. (1992): Diplosporous embryosac formation and the degree of diplospory in *Allium tuberosum. Sex. Pl. Reprod.* **5:** 72–78.

Kojima, A., Hinata, K. & Noda, S. (1991): An improvement of squash method for the cytological study of female meiosis in *Allium tuberosum*, Liliaceae. *Chromosome Inf. Serv.* **50:** 5–7.

Krantz, E., Boutor, I. & Lörz, H. (1991): *In vitro* fertilization of single isolated gametes of maize mediated by electrofusion. *Sex. Pl. Reprod.* **4:** 12–16.

Matzk, F. (1991): A novel approach to differentiated embryos in the absence of endosperm. *Sex. Pl. Reprod.* **4:** 88–94.

Mirochnichenko, E. (1964): Some biological specificity of *Poa* (to the methods of apomictic forms distinction). In: Cytology and selection of cultivated plants. *Nauka, Sib. Otd.* **2:** 49–58

Mirochnichenko, E. (1978): Facultative pseudogamous apomixis and caryological polymorphism in genus *Poa.* In: Apomixis in plants and animals. *Trudi biol. inst. Nauka, Sib.. Otd.* **35:** 224–236.

Naumova, T. (1990): Apomixis and amphimixis in angiosperms: Classification. *Apomixis Newslett.* **2:** 33–37.

Naumova, T.N. (1992): Apomixis in angiosperms: nucellar and integumentary embryony. 144. CRC, Boca Raton.

Naumova, T., den Nijs, A.P.M. & Willemse, M.T.M. (1992): Cytological approach to characterize apomixis in *Poa pratensis* genotypes. *Apomixis Newslett.* **4:** 31–34.

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

Oryol, L.J. (1986): Estimation of normal development of the cereal grains at early stages (based on new embryological technique). N.J. Vavilov Institute of Plant Industry, Leningrad, USSR.

Orlova, I.N. & Avalkina, N.A. (1985): Method of preparation of the whole embryo sacs of cultivated cereals. *Cytologia* **27**: 243–250.

Petrova, T.F. (1970): Technique of the endosperm total embryological slides preparing. *Bot. Zh.* **55:** 1662–1664.

Poddubnaja-Arnoldi, V.A. (1954): Time-saving techniques of embryological investigation of fixed material. *Bull. glavn. bot. Sada USSR* **18:** 81–91.

Rodkiewicz, B. (1967): Walls with callose in the megaspores and hypostase of ovules of *Antirrhinum majus* observed in a fluorescence microscope. *Bull. Acad. Pol.* **15:** 393–495.

Rodkiewicz, B. (1970): Callose in cell wall during megasporogenesis in angiosperms. *Planta* **93:** 39–47.

Solntseva, M.P. & Levkovskij, V.P. (1978): A rapid production of constant preparation of plant integral embryo sacs and pollen complexes. *Cytol. Genet.* **6:** 489–492.

Theunis, C.H., Pierson, E.S. & Cresti, M. (1991): Isolation of male and female gametes in higher plants. *Sex. Pl. Reprod.* **4:** 145–154.

Ulrich, I. & Ulrich, W. (1991): High-resolution flow cytometry of nuclear DNA in higher plants. *Protoplasma* **165:** 212–215.

Van der Maas, H.M., Zaal, M.A.C.M., de Jong, E.R., Krens, F.A. & van Went, J.L. (1993): Isolation of viable egg cells of perennial ryegrass. *Protoplasma* (submitted).

Van Went, J.L. & Kwee, H.S. (1990): Enzymatic isolation of living embryo sac of *Petunia. Sex. Pl. Reprod.* **3:** 257–262.

Verhoeven, H.A., Sree Ramulu, K. & Dijkhuis, P. (1990): A comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell suspension cultures of *Nicotiana plumbaginifolia. Planta* **182:** 408–414.

Wagner, V.T., Song, Y.C., Matthys-Rochon, E. & Dumas, C. (1988): The isolated embryo sac of *Zea mays*: structural and ultrastructural observations. In: Cresti, M., Gori, P. and Pacini, E. (eds): *Sexual Reproduction in Higher Plants*, 125–130. Springer, Heidelberg, Berlin, New York.