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New efforts to overcome apomixis in *Poa pratensis* L.

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Summary

By means of a new method, plants of *Poa pratensis* can be classified rapidly and reliably as to whether they are capable or incapable of parthenogenesis. Parthenogenesis was found to be under strong genetic control, dominant over obligatory fertilization. The selected sexual plants lack all genes/alleles responsible for parthenogenesis, while the polyploid apomictic varieties investigated were heterozygous with one or more dominant alleles. Also dosage effects and/or modifying genes are probably involved. Crosses of sexual individuals with various apomictic varieties resulted in sexual as well as highly apomictic F_1 hyrids. A scheme of recurrent hybridization for breeding of Kentucky bluegrass is proposed.

Two other experimental ways to overcome apomixis in *Poa pratensis* were studied in addition. By application of growth regulators temporary sexuality could not be induced. Attempts of *in vitro* regeneration of plants from endosperm resulted only in callus and root formation.

Introduction

Poa pratensis L., Kentucky bluegrass, is widely used for forage and turf. Most of the existing varieties of apomictic Kentucky bluegrass originated as individual plant selections collected from old turf areas or grasslands. For efficient breeding programmes the frequency of hybridization is too low in nearly all biotypes. Methods to manipulate apomixis are needed for future breeding progress.

In order to overcome apomixis several methods have already been tried or proposed, e.g., X-irradiation (Julén & Åkerberg, 1986), special crossing techniques (Funk & Han, 1967; Pepin & Funk, 1971, 1974; Riordan et al., 1988), somaclonal variation (McDonnell & Conger, 1984; Wu & Liu, 1985; Valk et al., 1988) and use of twins (Ostazeski et al., 1975; Den Nijs, 1990). However, in most cases breeders could not produce enough variation in their breeding stocks by the mentioned techniques. Therefore, the following three other strategies were studied to create variation via sexual recombination in Kentucky bluegrass. (1) Use of plants without parthenogenetic capacity. This way requires knowledge about the inheritance of parthenogenesis and an efficient method to distinguish 'sexual' and parthenogenetic individuals. (2) Induction of temporary sexuality by application of growth regulators. By means of induced temporary sexuality it would be possible to bring about recombination within and between apomictic varieties. (3) Regeneration of plants from endosperm. In the pseudogamous Kentucky bluegrass endosperms are formed only after fertilization of the polar nuclei, so as plants regenerated from endosperm would be hybrids. The presented paper describes the experiments and results of these three strategies.

Materials and methods

Strategy 1. For identification of plants being either incapable or capable of parthenogenesis, a new method was used (Matzk, 1991). It is based on the finding that in Gramineae a single application of a synthetic auxin at anthesis induces grain formation without fertilization. Both embryo and endosperm are lacking in such auxin-induced caryopses of sexual plants. Apomictic (parthenogenetic) plants, however, form caryopses with differentiated embryos and without endosperms (compare Fig. 1a and 1b). The auxins were applied by spraying panicles and leaves with, or dipping inflorescences into, aqeuous solutions of 2,4-D; 2,4,5-T; or dicamba in concentrations of 50 to 100 ppm.

Emasculation is not necessary in such tests if the auxins are applied immediately before anthesis. About 15 days after anthesis, or later, the developing caryopses can be examined under a dissecting microscope. About a hundred grains without endosperms were analysed per variant. Grains with endosperm, being the result of fertilization, were left out from analyses (Fig. 1a to 1c).

Five plants without and one with moderate parthenogenetic capacity (sexual clones), originating from the varieties 'Berbi', 'Prato', and 'Rožnovska', were selected and used for experiments to get some knowledge about the genetic control of parthenogenesis. The progenies (each with 20 to 50 plants) from open pollination, self pollination, or pollination of emasculated sexual clones with several other sexual or apomictic types were tested in field trials with 70 × 70 cm spaced plants. Crosses were made on potted plants in the greenhouse, using an optic magnifier for hand emasculation two days before anthesis. In the progenies the number of aberrant plants was counted and rated on 1–9 scale, with 1 being uniform and 9 highly variable. Chromosomes were scored in aceto-carmine stained male meiocytes.

Strategy 2. Growth regulators were applied for induction of temporary sexuality on potted plants of the varieties 'Alicja', 'Balin', 'Berbi', 'Delft', 'Erte', 'Hohenheimer', 'Leugra', 'Skandia 46'. The following agents (concentrations in ppm) were tested: Abscisic acid (100); adenine (80); 6-benzylaminonopurine (40, 80); 1-(4-chlorophenyl)-1,4-dihydro-4-oxo-6-methylpyridazine-3-carboxylic acid (2000, 5000); 3,6-dichloro-2-metoxybenzoic acid (50); 2,4-dichlorophenoxyacetic acid (60); thidiazauron (10); 2,3,5-triiodobenzoic acid (1000); zeatin (1).

Aqueous solutions of the agents were sprayed on panicles and leaves of plants from two to four varieties, between 16 and 4 days before anthesis. In order to estimate their effects on the seed formation system, the percentages of both parthenogenesis and aberrant plants were compared in progenies (each with 20 to 35 plants) of treated and untreated mother plants.

Strategy 3. For the experiments on *in vitro* regeneration of plants from immature endosperm, the varieties 'Berbi', 'Delft', 'Hohenheimer', and 'Skandia 46' were used. The tested media were:

MS (= Basal medium of Murashige & Skoog, 1962);
MS + 6 mg 2,4-D;
MS + 4 mg 2,4-D;
MS + 3 mg 2,4-D;
MS + 1 mg 2,4-D + 4 mg IAA + 0,2 mg kinetin;
MS + 0,1 mg GA_3 + 0,2 mg IAA + 0,5 mg BAP;
MS + 4000 mg yeast extract + 4 mg IAA + 2 mg kinetin.

Endosperm explants without embryos and hulls were tested between 14 and 27 days after pollination, with and without auxin pretreatment of panicles 6 to 10 days before excission.

Results

Some features connected with the seed formation system of eight clones, selected as 'sexual', are summarized in Table 1. All clones showed high segregation in their progenies. The clones S1 to S5 form seeds only after double fertilization (no par-

thenogenetic capacity) and twins are lacking. The clones S6 to S8, however, released high variability in progenies despite their high (S7 and S8) or medium (S6) parthenogenetic capacity and twin formation. Only S1 to S6 were used for analyses of the genetic control of parthenogenesis.

None of the 71 progeny plants resulting from self-pollination or crosses between the clones S1 to S5 (without parthenogenetic capacity) was parthenogenetic (Table 2). Open pollination or pollination of emasculated panicles with pollen from apomictic varieties yielded 99 hybrids without and 33 with high parthenogenetic capacity, besides 35 plants with a moderate degree of parthenogenesis. Such results may be expected if the mother plants (S1–S5) lack all genes/alleles, i.e. in all chromosome sets, necessary for parthenogenesis. The highly polyploid apomictic pollinators, on the other hand, were heterozygous, with one or more dominant alleles.

In self and open pollinated progenies of the clone S6 with moderate parthenogenetic capacity, most of the progeny plants showed again a medium degree of parthenogenesis but a few plants with high parthenogenesis or without parthenogenesis were also observed (Table 2). After crossing the clone S6 with sexual pollinators, plants with moderate as well as without parthenogenetic capacity arose frequently and only one hybrid was apomictic. Crosses with apomictic varieties, on the other hand, resulted in high frequencies of plants with moderate as well as with high parthenogenetic capacity besides a single sexual hybrid. Progeny plants with moderate parthenogenesis were often maternal (as a result of apomictic reproduction). The findings indicate that dosage effects and/or modifying genes are probably involved.

Results of F_2 and S_2 segregations have confirmed the above assumptions of genetic control (Table 3). Offsprings of hybrids with high parthenogenetic capacity were stable apomicts (e.g. S4 × A/1). Plants with parthenogenesis were never found in progenies of self-pollinated hybrids without parthenogenetic capacity (e.g. S3 × A/3, S3 SP/1, S4 × A/2). The correlation between the degree of parthenogenesis of the mother plants and the de-

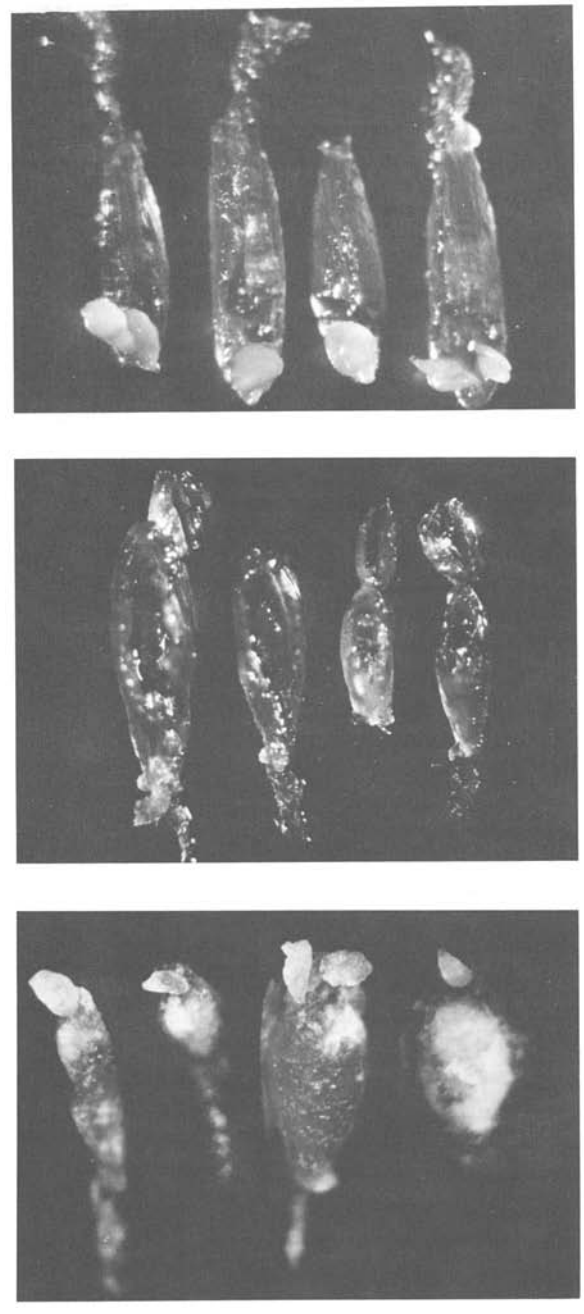

Fig. 1a–c. Immature caryopsis of *Poa pratensis* with and without capability of parthenogenesis (seed coat partly removed); a: auxin-stimulated caryopsis without endosperms and with embryos = parthenogenetic plant; b: auxin-stimulated caryopsis without endosperms and without embryos = sexual plant; c: caryopsis with endosperm and embryos (after pollination) = not useful for analysis.

Table 1. Characteristics of selected 'sexual clones' with and without parthenogenesis (mean values from three years)

Clone origin		Chromosome number (2n)	Degree of parthenogenesis, %	Frequency of polyembryony, %	Frequency of aberrants in progenies (1–9)
S1	Berbi	60	0	0	7
S2	Berbi		1,5	0	8/9
S3	Rožnovska	56	0	0	8
S4	Rožnovska	38	0,7	0	8/9
S5	Prato	49	0,5	0,3	7
S6	Berbi		38,0	3,6	6
S7	Lithuan.Pop.	60	91,7	2,4	6
S8	Lithuan.Pop.		76,6	3,0	6

gree of phenotypic segregation in the progenies was negative and highly significant ($r_S = -0.76$). Very strong inbreeding depression was observed in S_1 and S_2 progenies of all sexual plants.

In order to induce temporary sexuality by application of growth regulators, 95 experimental variants were tested. However, not change of the mode of seed formation in plants of highly apomictic varieties was observed. The frequency of off-types was never higher in progenies of treated than in those of control plants. Nevertheless, further experiments with other agents are under way.

For the attempts to regenerate plants *in vitro* from endosperm, immature caryopses were used. Callusses could frequently be induced from milk-waxy-ripe endosperms (about 16 days after pollination) on MS-medium supplemented with 3 or 4 mgl^{-1} 2,4-D. In some cases many fine roots developed (see Fig. 2). However, plants did not regenerate.

Discussion

The successful manipulation of apomixis in *Poa pratensis* by Julén (1960) and Grazi et al. (1961), or by Funk & Han (1967) and Pepin & Funk (1971) seems to be restricted to the specific genotypes used (varieties 'Fylking' and 'Bellevue'). A generally usable method to overcome the apomixis in *Poa pratensis* is not known up to date. For a breeding programme on the basis of cross-compatible sexual and apomictic individuals, a method is needed to identify sexual individuals rapidly and reliably.

Formerly, the mode of seed development was

Table 2. Parthenogenesis in progenies of five sexual clones (S1–S5) and one partially sexual clone (S6), after controlled pollination

Pollination of parents[a]	No. of progeny plants		Parthenogenesis (mean value)	Number of plants with parthenogenesis in %		
	Total	Analysed	%	0 to 8	12 to 48	50 to 97
S1–S5 (without parthenogenesis):						
EPP × S	118	25	1,2	25	0	0
SP	254	44	1,5	44	0	0
EPP × A	444	115	22,5	63	27	25
OP	369	52	16,8	36	8	8
S6 (with moderate parthenogenesis:						
EPP × S	100	13	21,1	6	6	1
SP	82	8	25,8	1	6	1
EPP × A	28	15	45,4	1	7	7
OP	88	11	25,8	3	7	1

[a] EPP: emasculated panicles pollinated, SP: self-pollination, OP: open pollination, S: sexual, and A: apomictic plants.

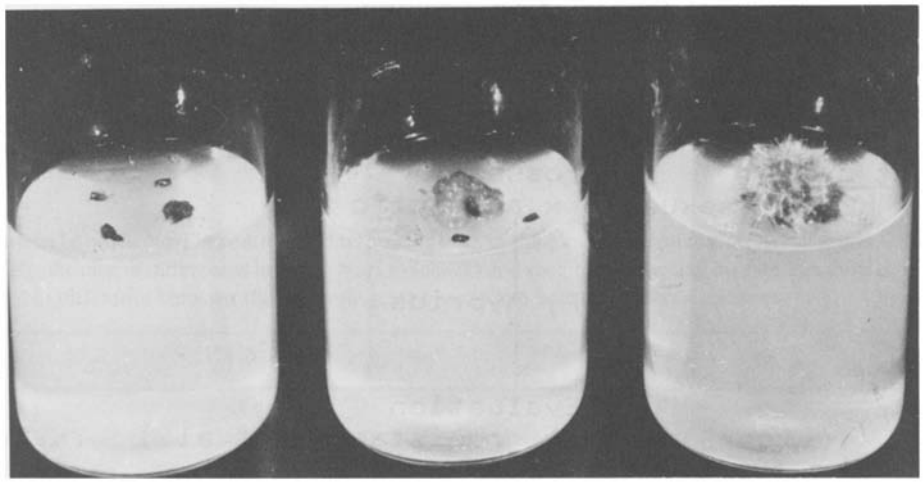

Fig. 2. Endosperm explants of *Poa pratensis* after about two months of culture (left: two darked explants without growth and two small callusses; middle: two darked explants without growth and one compact ivory-yellow callus; right: one big callus with many fine roots).

usually determined on the basis of the frequency of aberrant plants in space-planted progenies; all off-type individuals were regarded as to be of sexual origin. Such classification may be incorrect if autonomous development from reduced egg cells yields deviating polyhaploid plants ('haploid' parthenogenesis). In addition chimerism and somatic chromosomal instability of the highly polyploid genotypes may produce off-types. Results obtained with the clones S7 and S8 confirm these assumptions. The high frequency of aberrant plants in the progenies of S7 and S8 can not be interpreted as a result of hybridization since high parthenogenetic capacity of the parents was proved. In these cases the variability may have resulted from the mentioned reasons or other, unknown factors.

Also the method of embryo sac analysis proposed by Abelen et al. (1985) or Wilms (1985) for selection of sexual plants in *Poa pratensis* is, in our opinion, unreliable and too laborious. This method

Table 3. Phenotypic segregation and degree of parthenogenesis in F_2 and S_2 families of parents with different degrees of parthenogenesis (after self-pollination)

Mother plants		Progenies				
Origin[a]	Degree of parthenogenesis %	Phenotypic segregation (1–9)[b]	Parthenogenesis (mean value) %	Number of plants with parthenogenesis in %		
				0 to 8	12 to 48	50 to 97
S3 × A/1	65,1	1	54,2	0	2	3
S3 × A/2	22,6	8	10,1	5	5	0
S3 × A/3	2,8	9	0,6	18	0	0
S3 SP/1	0,4	8	1,5	8	0	0
S3 SP/2	8,4	8	0,9	21	0	0
S4 × A/1	73,1	2	57,3	0	0	3
S4 × A/2	1,4	8	0,4	9	0	0
S4 SP/1	2,9	6	2,8	1	0	0
S6 OP/1	30,0	6	35,8	0	5	0

[a] see Table 2.

[b] each progeny with 25 plants.

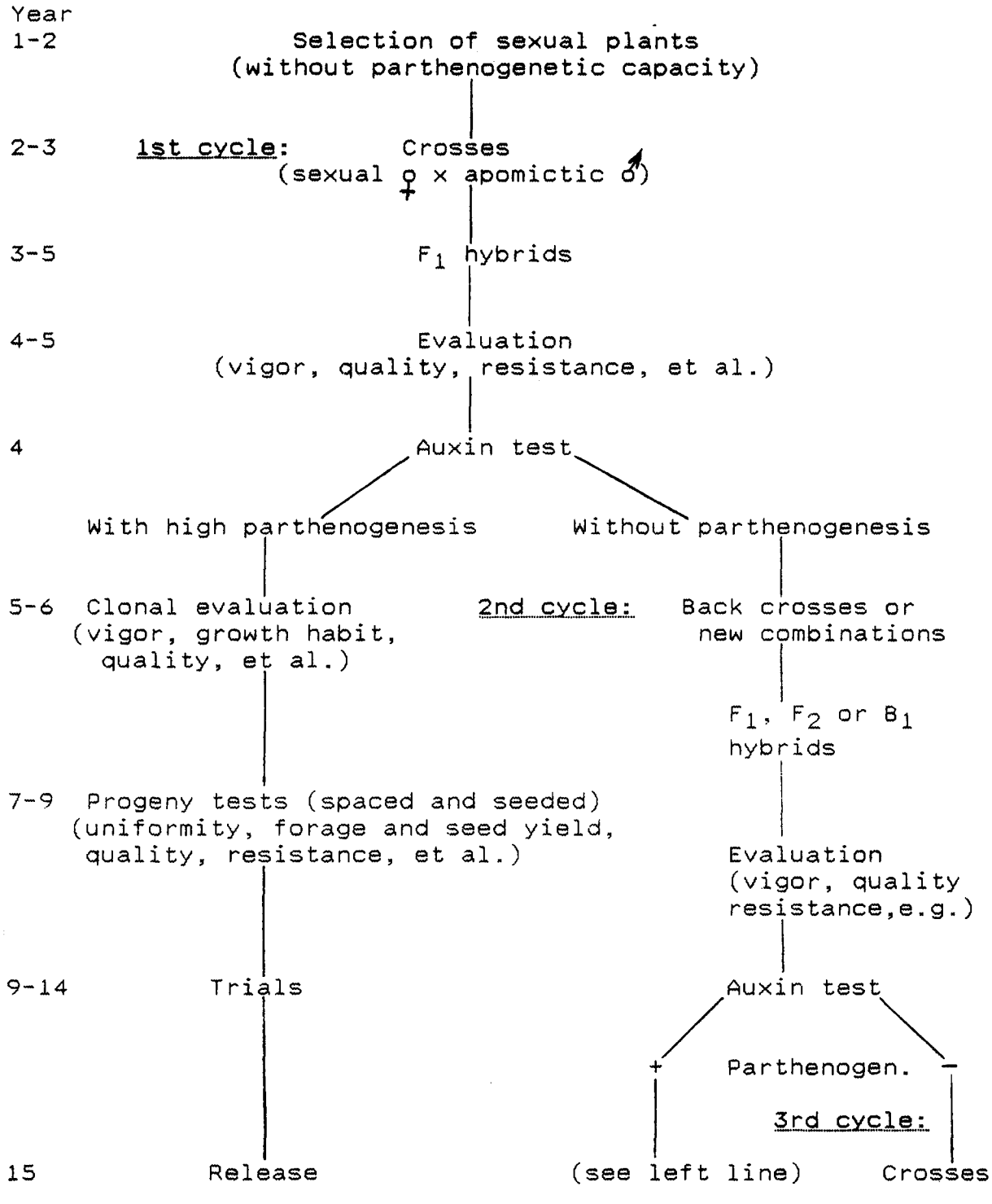

Fig. 3. A simplified breeding scheme with recurrent hybridization in *Poa pratensis* L.

only considers whether aposporous embryo sacs are formed or not, but the subsequent embryo formation is not been taken into account. These handicaps have been overcome by a recently developed method ('auxin test') for efficient identification of plants that are incapable of parthenogenesis (Matzk, 1988, 1991). Thus the five sexual clones S1 to S5, each of them forming embryos only after fertilization of the egg cells, could be selected by means of the 'auxin test' and used for studies on the inheritance of parthenogenesis.

Both the results on the genetic control of parthenogenesis and the availability of the 'auxin test' enables the author to recommend a cross-breeding scheme with sexual plants (Fig. 3). If using plants without parthenogenetic capacity, it may be taken for granted that all progenies are the result of fertilization. Further facilities of the 'auxin test' are: Emasculation is not necessary if auxin is applied before anthesis, and the analyses can be done only two weeks after the treatment or much later on stored panicles.

An exact and comprehensive genetic analysis is difficult in *Poa pratensis* because of high heterozygosity, high polyploidy, aneuploidy, chromosomal instability, and other reasons. Nevertheless, valuable results were obtained on the genetic control of parthenogenesis, for instance, the dominant expression of parthenogenesis over obligatory fertilization, the lack of genes/alleles for parthenogenesis in sexual plants, and the presence of one or more dominant alleles in the apomictic plants. In correspondence with these findings sexual as well as highly apomictic F_1 hybrids occurred in progenies of crosses between sexual clones and apomictic varieties. The results of the second cycle of hybridization are encouraging. Each cycle offers possibilities for selection of both promising apomictic hybrids for further evaluation and sexual plants with improved properties for new crosses (Fig. 3).

In the proposed programme, unfortunately, it is not possible to bring together characters and features from two apomictic varieties by direct crosses, as breeders would like. This aim should be reached via induced temporary sexuality. In our experiments with growth regulators, however, apomictic seed formation in *Poa pratensis* could not be changed or modified. On the other hand, the results on genetic control of parthenogenesis indicate, that contrary to the assumption by breeders, not only apomictic but also sexual segregants are to be expected after crossing two apomictic varieties.

The experiments of *in vitro* regeneration of plants from immature endosperms represent a novel approach to overcome apomixis. In the pseudogamous *Poa pratensis* the polar nuclei are fertilized. Therefore, the endosperms are a potential source for sexual recombination. Nakano et al. (1975), Bajaj et al. (1980), Sun & Chu (1981), Wang Ching-chu et al. (1982) and Zhao et al. (1984) have reported successful regeneration of single plants from endosperms in different cereals. All these studies were done in sexual species in connection with research on polyploidy. In our experiments, well growing callusses and in some cases even roots were obtained. Tests with the pseudogamous warm-season grasses *Panicum maximum* Jacq. and *Paspalum dilatatum* Poir. gave similar results. It was expected that in *Poa pratensis*, via an unstable callus phase only, plants with the common somatic chromosome number would regenerate from highly polyploid endosperm cells. Further extensive efforts are necessary in this field.

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