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Variation of microsporogenesis in sexual, apomictic and recombinant plants of *Poa pratensis* L.

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Abstract. Apomixis is a rather widespread phenomenon in plants. It is defined as the asexual formation of a seed from the maternal tissues of the ovule, avoiding the processes of meiosis and fertilization. Some species are facultative apomicts and form seeds by means of sexual and apomictic pathways to different extents. This is the case of *Poa pratensis*, the Kentucky bluegrass, which reproduces by aposporous pseudogamous facultative apomixis. This grass is one of the most studied apomictic systems, however some aspects, such as the male meiotic behavior, have not been so far investigated. In this study the process of microsporogenesis in genotypes of *P. pratensis* with a different mode of reproduction was investigated. The analysis revealed an almost regular meiosis in the sexual plants whereas apomictic genotypes exhibited different levels of meiotic irregularities, mainly due to cell fusion and irregular segregation in I and II division. Our data did not reveal evident connections between the extent and types of abnormalities and the components of apomixis, apomeiosis and parthenogenesis. The meiotic behavior of the examined plants was discussed in the light of their origin.

Keywords: *Poa pratensis* L., Kentucky bluegrass, apomixis, microsporogenesis, meiotic abnormalities.

INTRODUCTION

The sexual seed formation is based on two fundamental mechanisms, meiosis and fertilization. The combination of these events produces new nuclear compositions so that sexual reproduction is a means not only of generating new but also variable individuals. However, in some flowering plants, seeds form asexually, from maternal tissues by a process known as apomixis (Bicknell and Koltunow 2004). Apomixis, is a complex trait resulting from the circumvention of female meiotic reduction (a process known as apomeiosis) and fertilization (parthenogenesis). In gametophytic apomixis, a mega-

gametophyte (embryo sac) is originated from an unreduced cell, and subsequently a clonal embryo develops by parthenogenesis from a 2n egg (Matzk *et al.* 2005). Several species need fertilization for endosperm development while others do not (Barcaccia and Albertini 2013). The three components of apomixis, namely apomeiosis, parthenogenesis and autonomous endosperm formation, have been uncoupled experimentally, as documented in numerous genera such as *Taraxacum* (Van Dijk *et al.* 1999; Van Dijk, 2003), *Erigeron* (Noyes and Rieseberg 2000), *Poa* (Albertini *et al.* 2001), *Hypericum* (Barcaccia *et al.* 2006; Schallau *et al.* 2010), *Cenchrus* (Conner *et al.* 2013), and *Hieracium* (Catanach *et al.* 2006; Henderson *et al.* 2017).

Most plants of apomictic species are facultative apomicts and form seeds by means of sexual and apomictic pathways to different extents. This means that plants that reproduce by apomixis also retain the ability to reproduce sexually to varying degrees (Nogler 1994; Tucker 2003).

The phenomenon of apomixis is far from rare and its pattern of distribution suggests that it evolved many times during plant evolution. Among flowering plants it occurs with high frequency in certain families such as Asteraceae, Rosaceae, Ranunculaceae and Poaceae (Bicknell and Koltunow 2004).

Most apomictic plants produce viable pollen, this implies that within apomictic populations the formation of viable pollen represents a possibility for the fertilization of unreduced eggs. However, alterations of microsporogenesis in apomictic individuals have not been so far extensively investigated.

Poa pratensis L., Kentucky bluegrass, is an important fodder and turf grass which mainly reproduces by aposporous pseudogamous apomixis, *i.e.* unreduced aposporous embryo sacs develop through parthenogenesis to viable apomictic seeds if the unreduced polar nuclei fuse with a sperm cell from the male gametophyte (pseudogamy). The species is highly variable when reproduction mode, chromosome number and phenotypic traits are considered. In this species apomixis is facultative, with a frequency ranging from 0 to 100%, while chromosome numbers from 2n=18 to 150 have been reported (Matzk *et al.* 2005). Among the native monocot apomictic systems, *P. pratensis* is one of most explored. For several years, selected genotypes from wild Italian populations have been investigated with the aim of understanding the genetic control and mechanisms that regulate apomixis (Mazzucato 1995; Albertini *et al.* 2001; Porceddu *et al.* 2002; Raggi *et al.* 2015; Marconi *et al.* 2020). These studies provided a solid background for the present investigation that aimed at analyze the meiotic behavior of plants of *P. pratensis* exhibiting a different mode of reproduction and to find possible relationships between apomixis and its components and the alterations of microsporogenesis.

MATERIALS AND METHODS

Plant material

Genotypes of *P. pratensis* with different reproductive systems were examined: i) a sexual genotype S1/1-7 derived from a cross between two completely sexual genotypes selected from German cultivars (Matzk 1991); ii) an apomictic (aposporic and parthenogenetic) RS7-3 (Mazzucato 1995) and L4 (Marconi *et al.* 2020) plants, both from Italian natural populations and iii) several plants belonging to two F1 segregating populations produced by crossing S1/1-7 x RS7-3 (Barcaccia *et al.* 1998) and S1/1-7 x L4 (Marconi *et al.* 2020). Reproductive mode and chromosome number of the above reported materials employed in this study were investigated in previous studies (Barcaccia *et al.* 1998; Albertini *et al.* 2001; Porceddu *et al.* 2002; Marconi *et al.* 2020 and references therein) and are summarized in Table 1.

Plants were grown at the experimental field of the Dept. of Agricultural, Food and Environmental Sciences in Perugia (N 43°10'15.3'', E 12°39'58.7'').

Meiotic analysis

For meiotic investigations inflorescences not completely emerged from the flag leaf were employed. For each plant, four-five inflorescences were collected and immediately fixed in absolute ethanol-acetic acid 3:1 (v/v) for 24 hours, then they were transferred to 70% ethanol and stored at 4°C until analysis. Cytological preparations were made by squashing the anthers of a single flower on a glass slide with some drops of 0.5% acetocarmine (Merck Life Science, Italy), intensified by ferric oxide. For each plant 150-200 pollen mother cells (PMCs) for each meiotic stage were analyzed. Slides were observed under a Microphot Nikon microscope. Images were recorded with a digital photocamera SONY ICX282AQ and then processed using Adobe Photoshop 5.0. The alterations observed in each meiotic phase were expressed as percentage of the meiocytes examined.

For pollen viability analysis, pollen samples were collected from each plant, and stained with a mixture of acetocarmine and glycerol (1:1) (Ramanpreet and Gupta 2019). The pollen viability was expressed as percentage of fully stained pollen grains over a total of at least 1.000

Table 1. Name, progeny, mode of reproduction, somatic chromosome number (2n) with relative reference.

Name	Progeny	Mode of reproduction	2n	Reference for chromosome number determination
S1/1-7	-	Sexual	36	Porceddu *et al.* 2002
RS7-3	-	Apomictic*	64	Porceddu *et al.* 2002
L4	-	Apomictic*	42	Marconi *et al.* 2020
PG-$F_1$22	S1/1-7 ′ RS7-3	Sexual	50	Porceddu *et al.* 2002
PG-$F_1$15	S1/1-7 ′ RS7-3	Apomictic*	50	Porceddu *et al.* 2002
PG-$F_1$46	S1/1-7 ′ RS7-3	Apomictic*	50	Porceddu *et al.* 2002
PG-$F_1$5	S1/1-7 ′ RS7-3	Aposporic only	50	Porceddu *et al.* 2002
Apo143	S1/1-7 ′ L4	Aposporic only	39-42	Marconi *et al.* 2020
Apo40	S1/1-7 ′ L4	Parthenogenetic only	44-48	Marconi *et al.* 2020
Apo98	S1/1-7 ′ L4	Parthenogenetic only	39-42	Marconi *et al.* 2020

*Aposporic and parthenogenetic.

pollen grains for each sample. The percentage of the meiotic anomalies recorded at I and II division and pollen viability were graphically displayed.

RESULTS

Meiotic analysis of the parental genotypes

The sexual plant S1/1-7, with 2n=36, is considered a tetraploid with four additional chromosome pairs (Matzk 1991; Barcaccia *et al.* 1998; Porceddu *et al.* 2002); it exhibited an almost regular meiotic behavior with only few exceptions consisting of cell fusion and meiocytes linked by cytoplasmic connections at prophase I (4.3%). Pollen viability was almost complete reaching the 98.4% (Fig. 1a).

In the apomictic parental plant RS7-3 (2n=64), previously described as a probable octoploid having four additional chromosome pairs (Mazzuccato 1995; Porceddu *et al.* 2002), few abnormalities at different meiotic stages were observed. These included meiocytes at metaphase I with univalents (4.0%), anaphase I with lagging chromosomes (7.0%) and irregular segregation in the second division (15.0%). A high percentage of viable pollen was recorded (98.0%, Fig. 1b).

The apomictic L4 plant, hexaploid with 2n=42 (Marconi *et al.* 2020), revealed numerous abnormalities during both first and second division. At prophase I meiocytes linked by cytoplasmic connections (4.5%) were observed (Fig. 2a), whereas lagging chromosomes (19.0%) and irregular segregation (24.0%) were detected at anaphase I and II, respectively. Dyads and triads frequently occurred (15.0%) at the end of meiosis. The viable pollen produced by this plant was reduced to 51.0% with the grains displaying a quite heterogeneous size (Fig. 1c).

Meiotic analysis of F1 progenies

Four plants among those obtained from the cross S1/1-7 x RS7-3 were analyzed: sexual PG-$F_1$22, aposporic and parthenogenetic PG-$F_1$15 and PG-$F_1$46, and aposporic PG-$F_1$5. As showed by Porceddu and colleagues (2002) all these plants have a chromosome number 2n=50. The sexual plant PG-$F_1$22 showed an almost regular microsporogenesis with few exceptions consisting of anaphase I with laggards (8.6%), and few triads at telophase II (1.3%). The pollen viability was extremely high (99.0%, Fig. 1d). In PG-$F_1$15 events of cellular aggregation at prophase I (1.9%, Fig. 2b) and numerous cells at anaphase I with lagging chromosomes (50.0%) were found. In the second division, the irregular congression and segregation of chromosomes was observed in numerous meiocytes (15.0%). As a consequence, a considerable number of triads and dyads (13.0%) was produced at the end of meiosis. The pollen displayed variability in size but appeared fully stained (Fig. 1e). Genotype PG-$F_1$46 showed irregularities along the entire microsporogenetic process. These consisted in meiocytes at prophase I aggregated by cytoplasmic connections (1.4%), univalents at metaphase I (9.4%), lagging chromosomes at anaphases I (20.0%), and irregular orientation of chromosomes at metaphase II and anaphases II (24.0%). A conspicuous number of triads and dyads

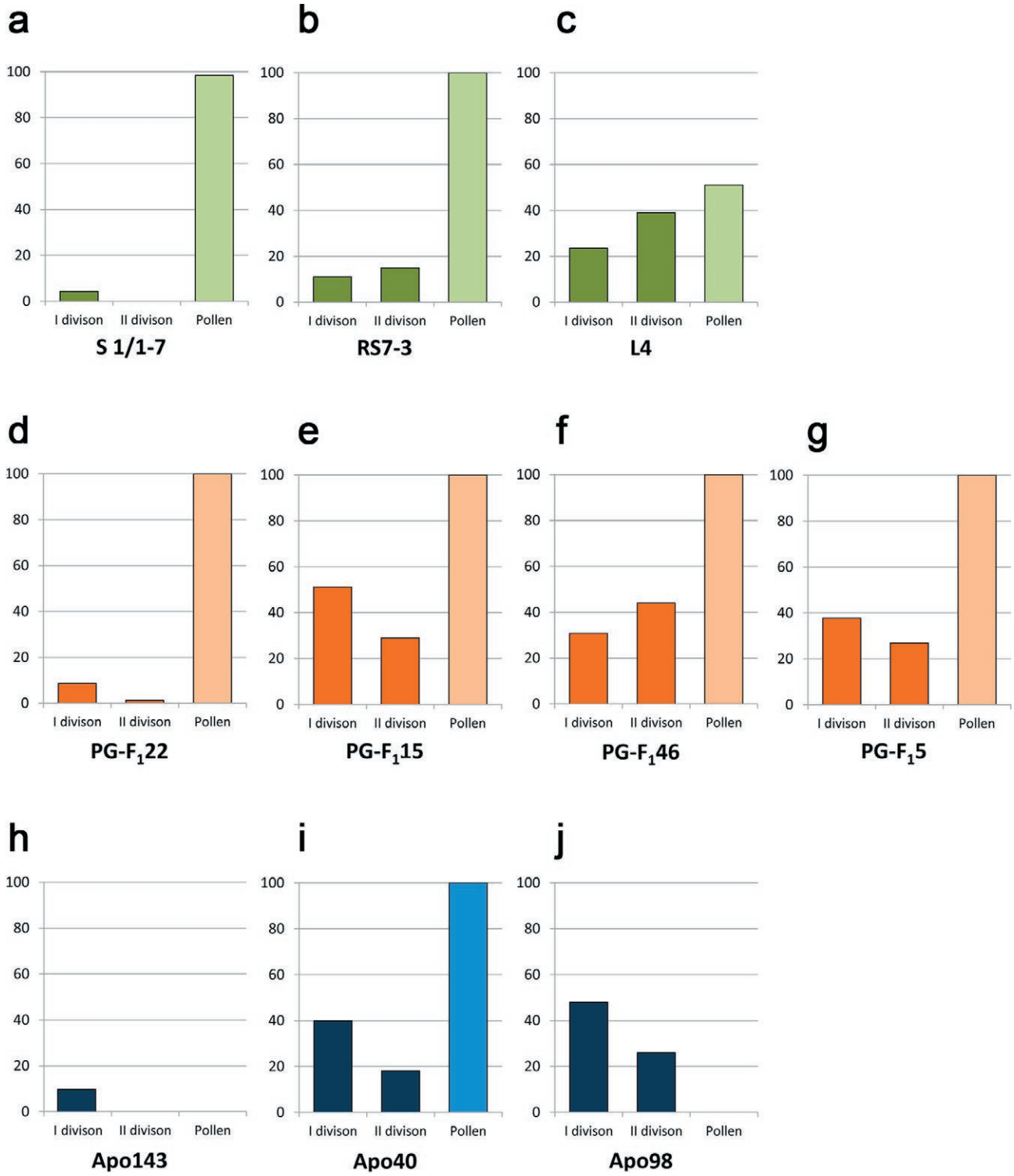

Figure 1. Percentage of meiotic abnormalities at I and II division and pollen viability recorded on parental genotypes S1/1-7 (**a**), RS7-3 (**b**) and L4 (**c**); on progenies from the cross S1/1-7 x RS7-3: PG-$F_1$22 (**d**), PG-$F_1$15 (**e**), PG-$F_1$46 (**f**), PG-$F_1$5(**g**) and from the cross S1/1-7 x L4: APO 143 (**h**), APO 40 (**i**) and APO 98 (**j**).

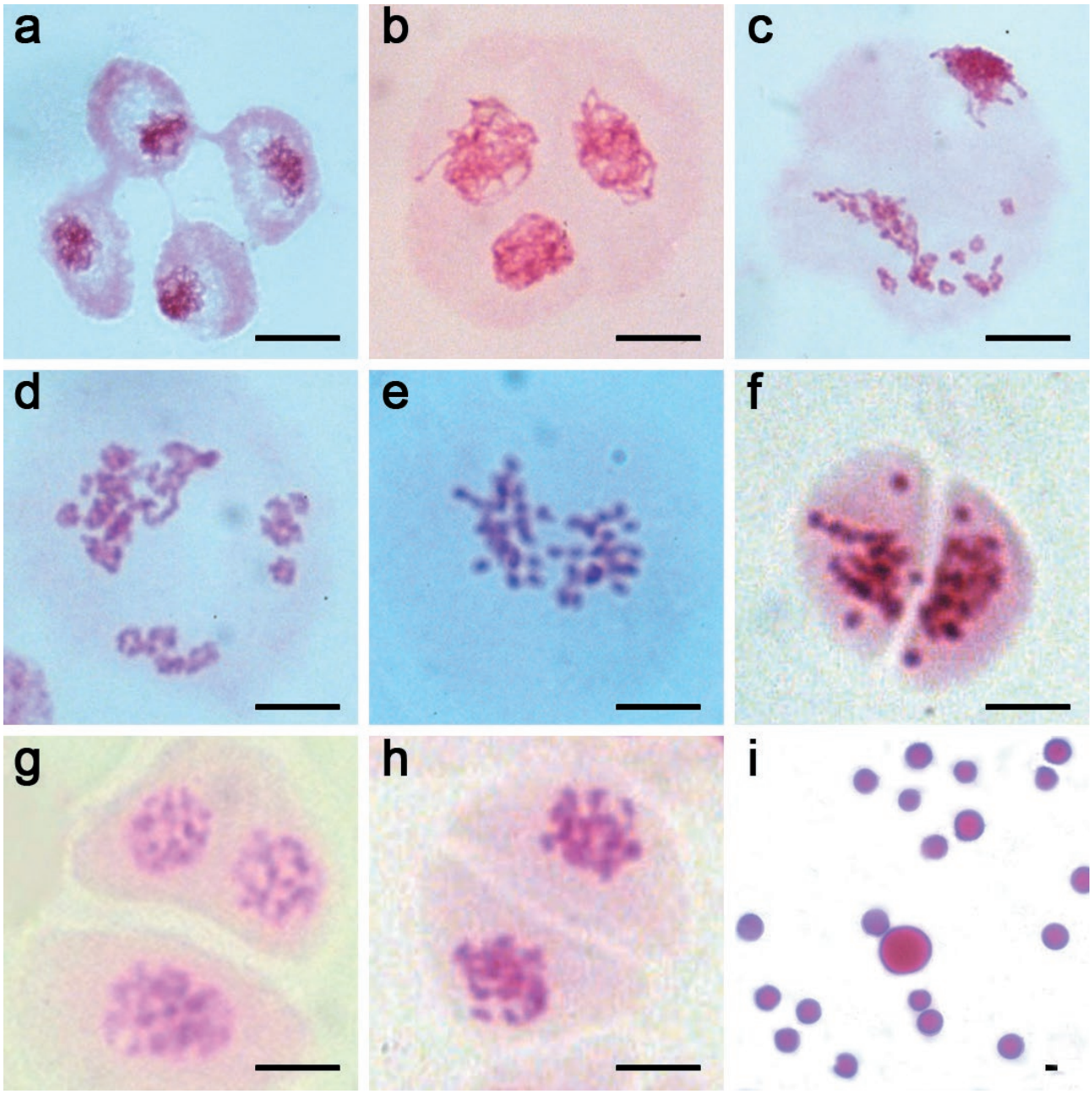

Figure 2. Aspects of alterations of microsporogenesis and pollen grains in some of the examined genotypes. Meiocytes at prophase I connected by cytoplasmic channels in L4 (**a**); Fusion of three cells at the beginning of prophase I in PG-F115 (**b**); fusion of two meiocytes at different stage of prophase I in PG-F15 (**c**); cell at the diakinesis stage with chromosomes separated into three groups (**d**); absence of chromosome segregation at anaphase I in PG-F15 (**e**); spreading of chromosomes in both cells of a meiocyte at anaphase II in PG-F15 (**f**); triad formed at the end of microsporogenesis in APO 40 (**g**); dyad formed at the end of microsporogenesis in APO 40 (**h**); pollen grains produced by APO 40 exhibiting a large size variability (**i**). The bar represents 10 μm.

was recorded at the end of meiosis (20.0%). A considerable variability of the pollen size was observed; however, pollen viability of this plant was complete (Fig. 1f). The aposporic recombinant PG-$F_1$5 displayed a quite anomalous microsporogenesis. At prophase I, fusion and aggregation of meiocytes (4.7%) were observed, with fusions that in some cases involved meiocytes at prophasic stages (Fig. 2c). In addition, cells with chromosomes sepa-

rated into two or three groups were observed (Fig. 2d). At anaphase I, numerous PMCs (33.0%) showed lagging chromosomes or absence of segregation (Fig. 2e). In the second division the spreading of chromosomes in one or both cells of meiocytes was frequently observed (15.0%, Fig. 2f), as well as dyads and triads at the end of meiosis (12.0%). Despite these abnormalities, also in this case the pollen appeared fully viable (Fig. 1g).

Among the progeny of the cross S1/1-7 x L4, the aposporic APO143 and parthenogenetic APO40 and APO98 were analyzed. The chromosome numbers of these genotypes, 2n=44-48 for APO40 and 2n=39-42 for APO98 and APO143, was previously estimated by means of flow cytometry (Marconi *et al.* 2020). In APO143 most of the anthers resulted empty and the few meyocites that was possible to analyse showed anomalies consisting of linked cells (4.5%) at prophase I and lagging chromosomes at anaphase I (5.0%). The few pollen grains produced, were not viable (Fig. 1h). The meiotic irregularities detected in APO40 mainly affected the first division where about 6.0% of PMCs at prophase I were connected by cytoplasmic channels and 34.0 % of meiocytes at metaphase I showed univalents. Anomalies of the second division were due to the absence of segregation of chromosomes at anaphase II (8.0%) and formation of triads and dyads (9.0%) at the completion of meiosis (Fig. 2g, h). The pollen showed a remarkable variability in size but all grains appeared fully stained (Fig. 1i, Fig. 2i). In APO48 a high number of cells at anaphase I with laggards were recorded (48.0%). The scarcity of meiocytes in the second division suggests a possible degeneration of PMCs before the dyad stage. A remarkable number of cells that entered the second division displayed irregular segregation of chromosomes (26.0%) and the pollen was not viable (Fig. 1j).

DISCUSSION

In this work the meiotic behavior of sexual, apomictic and F_1 recombinant genotypes of *P. pratensis* was investigated. In sexual plants an almost regular microsporogenesis was observed, whereas the apomictic genotypes displayed meiotic abnormalities at different degrees, mostly consisting in events of cell fusion and irregular segregation of chromosomes during I and II division. Data did not reveal relationships between the amount and types of such abnormalities and the reproductive mode of the apomictic genotypes. However, the origin of the parental genotypes S1/1-7, RS7-3 and L4 may offer useful indications for interpreting their meiotic behavior and those of their progenies. In fact, the sexual S1/1-7 plant was derived from a cross between two completely sexual genotypes selected from German cultivars (Matzk 1991; Barcaccia *et al.* 1998). The achievement and persistence of sexuality by means of meiosis and fertilization is guaranteed by regular processes of microsporogenesis and gametogenesis. The fact that S1/1-7 was obtained by crossing two completely sexual genotypes contributed to preserving its fertility. Conversely, the apomictic RS7-3 and L4 were collected in the wild and did not undergo any anthropogenic pressure (Mazzuccato 1995; Marconi *et al.* 2020). These genotypes differ in chromosome number, meiotic behavior and pollen fertility, and also their progenies, obtained by crossing them with S1/1-7 as female parent, showed variability in the same traits. Given that both crosses have the same female parent, it is possible to evaluate the contribution of each male parent to the characteristics of the corresponding offspring. Since all plants from the cross S1/1-7 x RS7 had the same chromosome number 2n=50 (Porceddu *et al.* 2002) this evidence demonstrates that a regular chromosome segregation occurred and that fertilization took place between normal haploid female and male gametes with n=18 and n=32, respectively. On the contrary, two different ranges of chromosome number (2n=39-42 and 2n=44-48) were detected in plants from S1/1-7 x L4 (Marconi *et al.*, 2020). This suggests that L4 produced functional gametes with a different chromosome number and that they accomplished fertilization.

It has been suggested that the meiotic events are controlled by a large number of genes, some controlling the meiotic phases and others post-meiotic events and gametogenesis. The mutation of any of these genes can cause anomalies affecting the gamete fertility (Ma 2005).

Most of the meiotic abnormalities observed in this study were common to all plants examined; for example, the irregular segregation of chromosomes, which is probably due to the defective spindle formation or its total absence (Kaul and Murthy 1985). Generally, these alterations are not directly responsible for pollen viability but rather for pollen chromosome number; so that they are considered one of the principal sources of polyploid or aneuploid-polyploid pollen grains (Stebbins 1963; Podio *et al.* 2012). This may explain why, despite their meiotic disturbances, RS7-3 and the apomictic and recombinant progenies PG-$F_1$5, PG-$F_1$15 and PG-$F_1$46 showed a high level of pollen fertility. The considerable number of triads and dyads and the heterogeneous size of pollen detected in PG-$F_1$15 and PG-$F_1$46 are a further evidence that these meiotic alterations influence the chromosome constitution of pollen grains (Stanley and Linskens 1974).

The genotype L4 as well as genotypes APO143, APO40 and APO98 displayed a different situation. The

meiotic alterations detected in L4 could explain the production of aneuploid pollen grains, as above suggested, but do not clarify the cause of the reduction in pollen fertility of this plant. This, most likely, is the result of post-meiotic events, such as the alteration of the normal activity of genes controlling the steps following the completion of meiosis and gametogenesis (Lalanne and Twell 2002).

The scarcity of PMCs detected in APO143 could be the consequence of mutations affecting the normal development of anthers and the formation of meiocytes. A certain degree of PMCs scarcity has been already reported in *Boechera* (Rojek *et al.* 2018) while genetic and molecular analyses in *Arabidopsis* demonstrated that a high number of genes controls several aspects of anther development and that their mutations can seriously damage the anther cell differentiation, tapetum function and microspore development (Ma 2005; Sanders *et al.* 1999).

Studying mutants in Arabidopsis, Yang and colleagues (2003) demonstrated that mutations of genes controlling the meiotic progression can result in programmed cell death with the consequence of the death of most meiocytes before cytokinesis. The dramatic reduction in the number of meiocytes observed in APO98 could be the result of a phenomenon of cell degeneration similar to the one described in Arabidopsis.

Among those obtained from the cross S1/1-7 x L4, the parthenogenetic recombinant APO40 was the only genotype producing viable pollen. Moreover, the microsporogenesis pathway of this plant was not affected by the scarcity of meiocytes that characterized APO143 and APO98. A possible explanation for the different meiotic behavior of the F_1 plants from the cross S1/1-7 x L4 could be the different number of mutations that these plants inherited from the male parent; the different chromosome number characterizing these genotypes could support this hypothesis.

Further considerations can be done taking into account the cytological data and the reproduction mode of the plants obtained from the cross S1/1-7 x RS7. It can be observed that the apomictic and recombinant genotypes have similar behavior as the male parent, whereas the sexual PG-$F_1$22 reflects the meiotic characteristics of the female parent. This suggests that the meiotic behavior and the mode of reproduction are together inherited from one of the parents. However, the progeny from S1/1-7 x L4 does not provide enough evidence supporting this hypothesis because all the examined genotypes were apomictic recombinant.

Cellular aggregations due to cytoplasmic channels and cell fusion represent sporadic events in the examined plants. Such aggregations involved only few cells (2-4) and did not damage the pollen fertility because they did not proceed to meiosis but degenerated, as demonstrated by the fact that they were not observed from prophase I onwards. Cell fusion has been reported in several plant species and may result from suppression of cell wall formation during premeiotic mitoses (Nirmala and Rao 1996). Instead, the cytoplasmic connections originate from the pre-existing system of plasmodesmata which form within anther tissues and subsequently become completely obstructed by the progressive deposition of callose (Heslop-Harrison 1966). In some cases, due to the scarce production of callose, the connections remain giving origin to meiocytes aggregation. The discovery of this phenomenon in *P. pratensis* is interesting because it has been demonstrated that the defective deposition of callose is a critical step in the anomalous development of female gametophyte in apomictic plants (Peel *et al.* 1997; Dusi and Willemse 1999).

Further investigations based on a higher number of samples could clarify the hypotheses made in this study. Considering that the fertility of plants is a complex mechanism, it would be useful to combine the meiotic analysis with the analysis of the reproductive structures and, in particular, of the anthers.

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