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Megagametophyte organization in diploid alfalfa meiotic mutants producing 4n pollen and 2n eggs

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Abstract Megagametogenesis was studied in five diploid alfalfa mutants producing 4n pollen and 2n eggs, using a stain-clearing technique. All mutants produced embryo sacs with a variable number of supernumerary nuclei both at the early (bi- and tetra-nucleate) and at the late (eight-nucleate) stages of development. The presence of supernumerary nuclei is considered to be a consequence of the production of coenocytic megaspores. The production of 2n eggs was confirmed through cytological investigation by means of the diameter of the egg-cell nucleolus. The frequency of 2n eggs was lower than the frequency of binucleated macrospores as previously determined. This discrepancy may be due to environmental effects but also to the fact that binucleated macrospores may degenerate or may, after two mitotic divisions, give rise to eight-nucleated embryo sacs counted as normals.

Key words Alfalfa · Megagametogenesis · Supernumerary nuclei · 2n eggs · Meiotic mutants

Introduction

In the *Medicago sativa* complex a meiotic mutation causes the production of 4n pollen ("jumbo pollen"). Previous research has established that this mutation is due to a single recessive gene, named "*jp*", which causes the failure of postmeiotic cytokinesis during microsporogenesis (McCoy and Smith 1983; Pfeiffer and Bingham 1983). Jumbo pollen mutants behave essentially as male sterile, but they have normal female fertility and can also produce a high frequency of 2n eggs (McCoy and Smith 1983). Therefore, they can be used as mother plants to obtain tetraploids via unilateral or bilateral sexual polyploidization. During a selection programme aimed at increasing the frequency of 2n gametes in an experimental diploid population of alfalfa, five plants producing jumbo pollen and 2n eggs at high frequency were identified (Veronesi et al. 1990). An analysis of micro- and macro-sporogenesis performed on these plants revealed that they produced tetranucleated microspores and jumbo pollen due to the failure of the post-meiotic cytokinesis, and bi-, tri- and tetranucleated macrospores due to the lack of cytokinesis after the first- and/or second meiotic division. The fusion of nuclei in the binucleated macrospores resulted in the production of 2n macrospores of the SDR type, which were recognized on the basis of nucleolar dimensions. Observations on clone H25 indicated that it produced embryo sacs with supernumerary nuclei (Mariani et al. 1993). Based on these findings the present research was conducted: (1) to verify the production of macrogametophytes (MGs) with supernumerary nuclei in all five *jp* clones; (2) to determine if such abnormal MGs derive from coenomegaspores; and (3) to further confirm the production of 2n eggs through cytological investigation.

Materials and methods

The jumbo pollen plants analyzed (H21, H23, H25, H27, H29) are the same as reported in a previous study (Mariani et al. 1993). Buds of these plants were fixed in FAA and ovules were prepared according to the technique proposed by Stelly et al. (1984) which proved effective in observing alfalfa micro- and macro-sporogenesis (Tavoletti et al. 1991). The analysis of the MGs developmental stages was performed on 1 482 ovules, ranging from 324 in H29 to 466 in H23. Unreduced egg cells were identified by their nucleolar dimensions. For this purpose 547 of the 1 482 ovules were used. The nucleolar-size distributions of *jp* plants were compared to those of diploid and tetraploid controls (100 measurements each).

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Table 1 Different types of MGs in the *jp* clones

Jp clones	Number of MGs with 8–16 nuclei								No. of aborted	Total no.
	8	8[a]	9	10	11	12	13	16	MGs	of MGs
H21	269	0	1	1	0	2	0	0	63	336
H23	260	1	2	0	2	0	1	0	78	344
H25	303	2	4	1	0	0	0	0	20	330
H27	223	3	2	3	1	2	0	0	32	266
H29	171	2	3	2	0	0	0	1	27	206

[a] 8: MGs with two polar nuclei of different sizes

Results

In alfalfa, normal macrospores develop into eight-nucleate embryo sacs of the *Polygonum* type. The three antipodal cells degenerate early, and the two polar nuclei fuse completely at the time of fertilization (Reeves 1930; Cooper 1935). In the five jumbo pollen clones analyzed normal MGs were found together with MGs with supernumerary nuclei and degenerating MGs. The number of nuclei found in these abnormal MGs ranged from 9 to 16 (Table 1 and Fig. 1A, B.). In some of these MGs three polar nuclei were evident (Fig. 1 C). Macrogametophytes with two polar nuclei of different sizes were also observed in all of the clones except H21 (Fig. 1 D, E and Table 1), with the larger nucleus probably being derived from the fusion of two. In Table 2 the number of nuclei in the micropylar and chalazal ends and in the central region of abnormal MGs is reported. It is significant that only one supernumerary nucleus was found in the central region, whereas more than one supernumerary nucleus was observed at the micropylar and/or chalazal ends. Furthermore, one MG with only two nuclei at the chalazal end, instead of the normal three, was found in clone H25. In a few cases, mature ovules with two MGs at different developmental stages were observed. Most of these MGs were at early stages of development, except in one case (clone H21) in which a mature MG had formed together with a tetranucleated one (Fig. 1 F–J); ovules with two embryo sacs probably give rise to polyembryonic seeds. In fact, in the jumbo pollen clone progenies from 2x-2x crosses the presence of at least one polyembryonic seed was ascertained (Fig. 1 K). Furthermore, MGs at early developmental stages were found in ovules in which, judging by their size, mature MGs would have been expected (Fig. 1 L). Some of these MGs had a very large vacuole, indicating that they would be unable to develop any further. The early stages of development were also examined in the five *jp* mutants. In all of them, except clone H23, MGs with six nuclei of the same size were found in ovules in which bi- and tetra-nucleated MGs are normally present (Fig. 1 M–Q,). MGs with five nuclei were observed in the same kind of ovules (Fig. 2A–E and Table 3). In all five *jp* clones, n and 2n bi- (Fig. 2 F–I) and tetra-nucleated MGs were identified on the basis of their nucleolar dimensions. In fact, the nucleolar size of these MGs was comparable to that of bi- and tetra-nucleated MGs from diploid and tetraploid plants, respectively. In some MGs, divisions of embryo-sac nuclei were observed, occasionally with asynchrony between the micropylar and chalazal poles (Fig. 2 J,K). This phenomenon is common in alfalfa MG development (Reeves 1930). Asynchronous divisions between the two poles most probably lead to the formation of MGs with six nuclei of different sizes (Fig. 2 L–O), as was recorded in birdsfoot trefoil (Rim et al. 1990). It is significant that in clone H27 a MG with eight mitotic spindles was present (Fig. 2 P–T), which could give rise to a 16-nucleated stage, as was observed in clone H29.

In order to confirm the production of 2n eggs, the nucleolar diameters of egg cells were measured in the five jumbo pollen mutants and in diploid and tetraploid controls. The frequency distributions of the *jp* mutants covered the whole range defined by the controls, showing that the mutants produce 2n eggs. The frequency of 2n eggs was determined (Fig. 3) according to the mathematical system previously used to calculate the proportion of n and 2n pollen in a given plant (Veronesi et al. 1988). The presence of 2n and 4n egg cells was not taken into account as very large cells occurred only in very few cases.

Table 2 Distribution of nuclei in the micropylar, central and chalazal regions of abnormal MGs

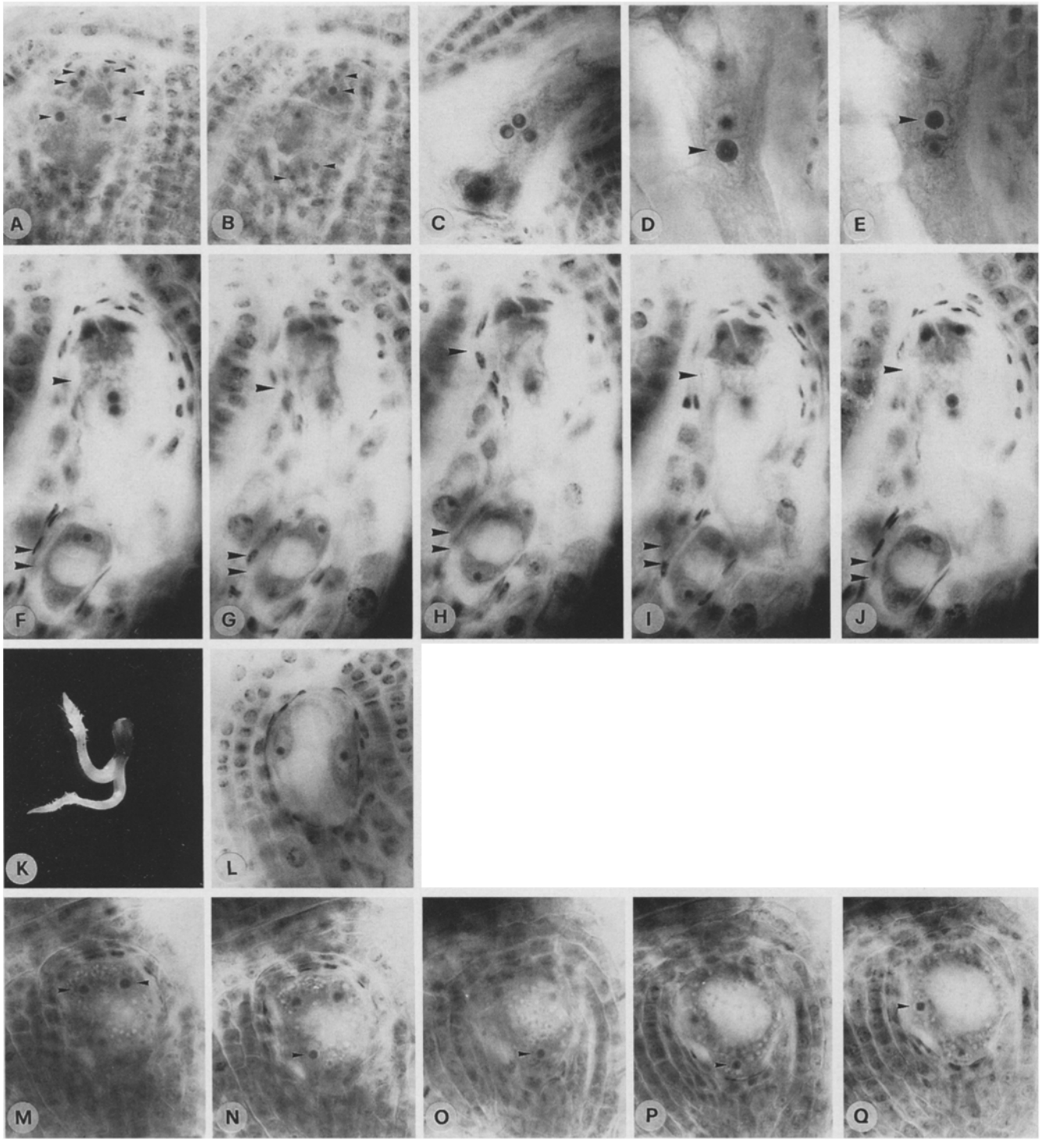
JP clones	Number of MGs with 3–12 micropylar nuclei						
	3	4	5	6	7	8	12
H21	0	2	0	2	0	0	0
H23	2	0	2	1	0	0	0
H25	2	0	0	2	0	1	0
H27	1	0	4	1	2	0	0
H29	2	1	1	0	1	0	1

	Number of MGs with 0–3 polar nuclei			Number of MGs with 0–5 chalazal nuclei				
	0	2	3	0	2	3	4	5
H21	0	1	3	0	0	4	0	0
H23	0	2	3	0	0	4	0	1
H25	1	0	4	2	1	2	0	0
H27	0	6	2	0	0	8	0	0
H29	2	1	3	1	0	2	3	0

Table 3 Number of nuclei in MGs of ovules at early stages of development

JP clones	Number of MGs with 2–6 nuclei				
	2	3	4	5	6
H21	54	0	38	0	1
H23	69	2	49	2	0
H25	30	2	48	1	3
H27	43	1	33	1	1
H29	60	2	48	3	5

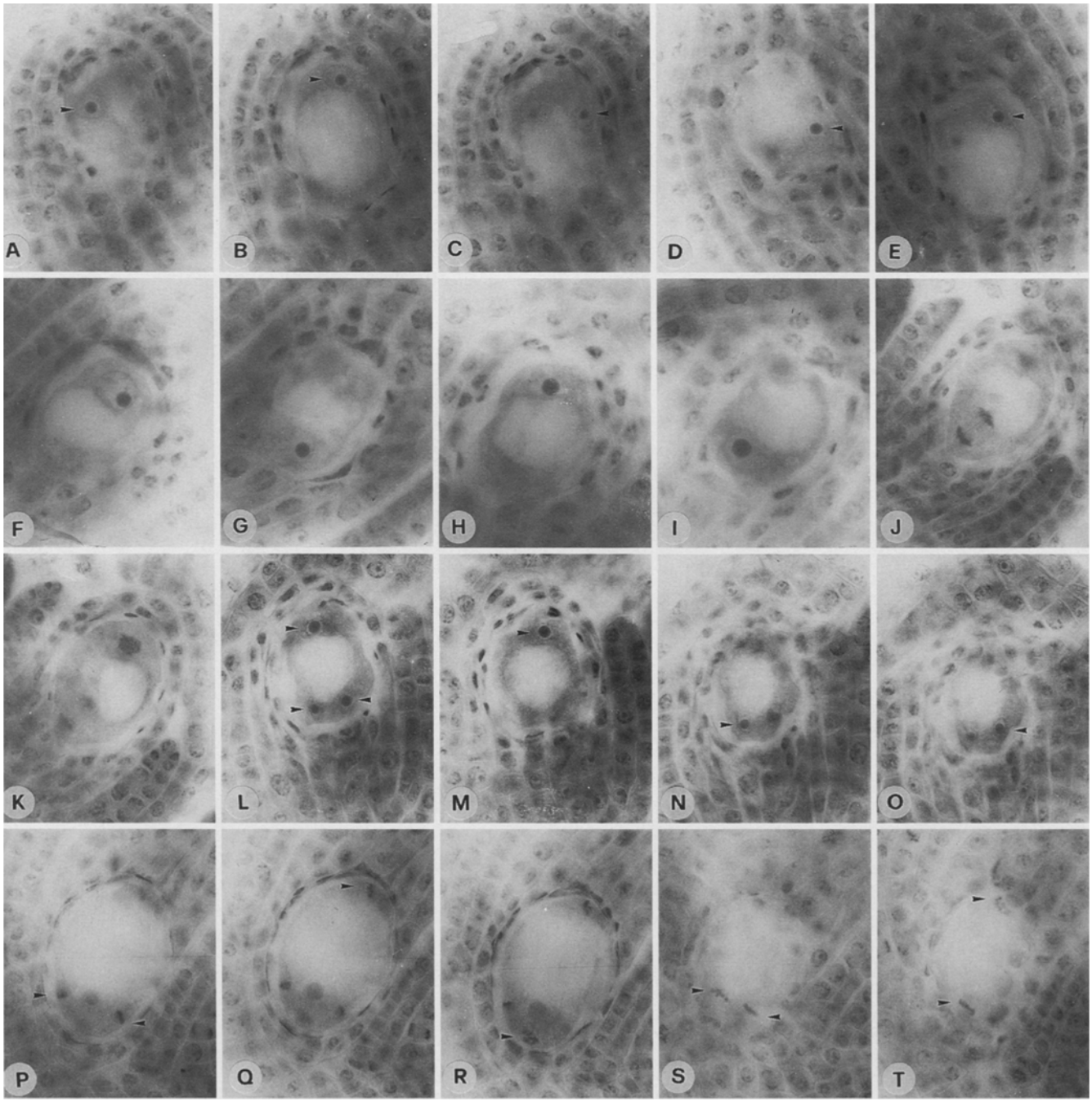
Fig. 1A–Q **A, B** Clone H25, macrogametophyte (MG) with ten nuclei (*arrows*, x 925). **C** Clone H27, MG with three polar nuclei (x 925). **D, E** Clone H25, MG with two polar nuclei of different sizes (*arrows*, x 1 480). **F–J** Clone H21, an ovule with a mature (*single arrow*) and a tetranucleated (*double arrow*) MG (x 1 180). **K** Polyembryonic seed in clone H29 progenies (x 690). **L** Clone H21, binucleated MG in a mature ovule (x 925). **M–Q** Clone H25, MG with six nuclei of the same size (*arrows*, x 925)

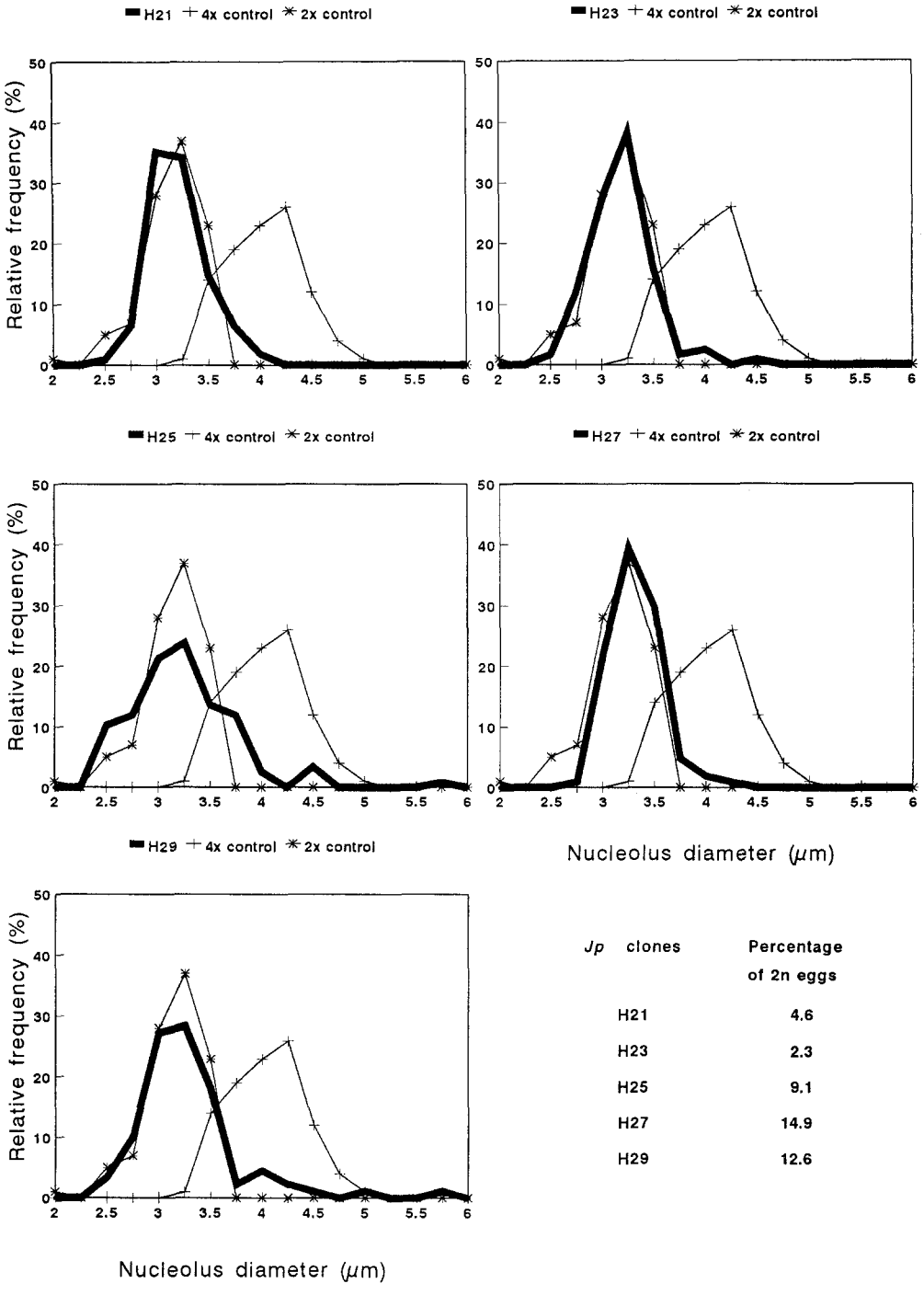


Discussion

The presence of supernumerary nuclei in the dicotyledonous female gametophyte is not very common. Kennell and Horner (1985) observed that the *ms1* male-sterile mutant of soybean produced MGs with more than eight nuclei as a result of the formation of coenomegaspores. In a haploid-diploid twin-producing polyembryonic line of *Linum usitatissimum* Secor and Russell (1988) found MGs with one or two supernumerary egg cells. They postulated such supernumerary cells as the originators of the haploid embryos produced by the line. However, they did not investigate megasporogenesis to verify the production of coenomegaspores. Our results, in agreement with previous findings by McCoy (cited by Kennel and Horner 1985), showed that alfalfa jumbo pollen plants produce embryo sacs with supernumerary nuclei and 2n egg cells. It is presumed that these embryo sacs derive from coenocytic

Fig. 2A–T **A–E** Clone H23, MG with five nuclei (*arrows*, x 925). **F–I** n (**F, G**) and 2n (**H, I**) MGs (x 1 480). **J, K** Clone H29, MG with an anaphase at the chalazal pole and a prophase at the micropylar pole (x 925). **L–O** Clone H29, MG with six nuclei of different sizes (*arrows*, x 925). **P–T** Clone H27, MG with eight mitotic spindles (*arrows*, x 925)





Jp clones	Percentage of 2n eggs
H21	4.6
H23	2.3
H25	9.1
H27	14.9
H29	12.6

Fig. 3 Frequency distributions of egg-cell nucleolar diameter in the *jp* clones and in 2x and 4x controls. The percentage of 2n eggs in the *jp* clones is also given

megaspores. In fact, bi-, tri- and tetra-nucleated macrospores have already been found in the same mutants (Mariani et al. 1993). In Fig. 4 some of the different patterns of development of coenocytic megaspores are reported, assuming the fusion and the division of a variable number of nuclei. In the present study, MGs with five and six nuclei were found in ovules at early stages of development, which could only derive from multinucleated macrospores. In fact the six-nucleate MG may result from a trinucleated macrospore after the first mitotic division, and the five-nucleate MG may result from the same kind of macrospore in which one of the three nuclei does not undergo the first mitotic division. The three-nucleate MGs may derive directly from the vacuolization of a three-nucleate macrospore and give rise to the five- and six-nucleate MGs previously mentioned. As reported in Fig. 4, five- and six-nucleate MGs may also derive from a binucleated macrospore after two mitotic divisions in which one or more nuclei are not involved, admittedly a more complicated, and therefore hardly convincing, hypothesis. The origin of five- and six-nucleate MGs from a tetranucleated macrospore seems even less probable, since it would imply that only one or two nuclei have divided, and especially as

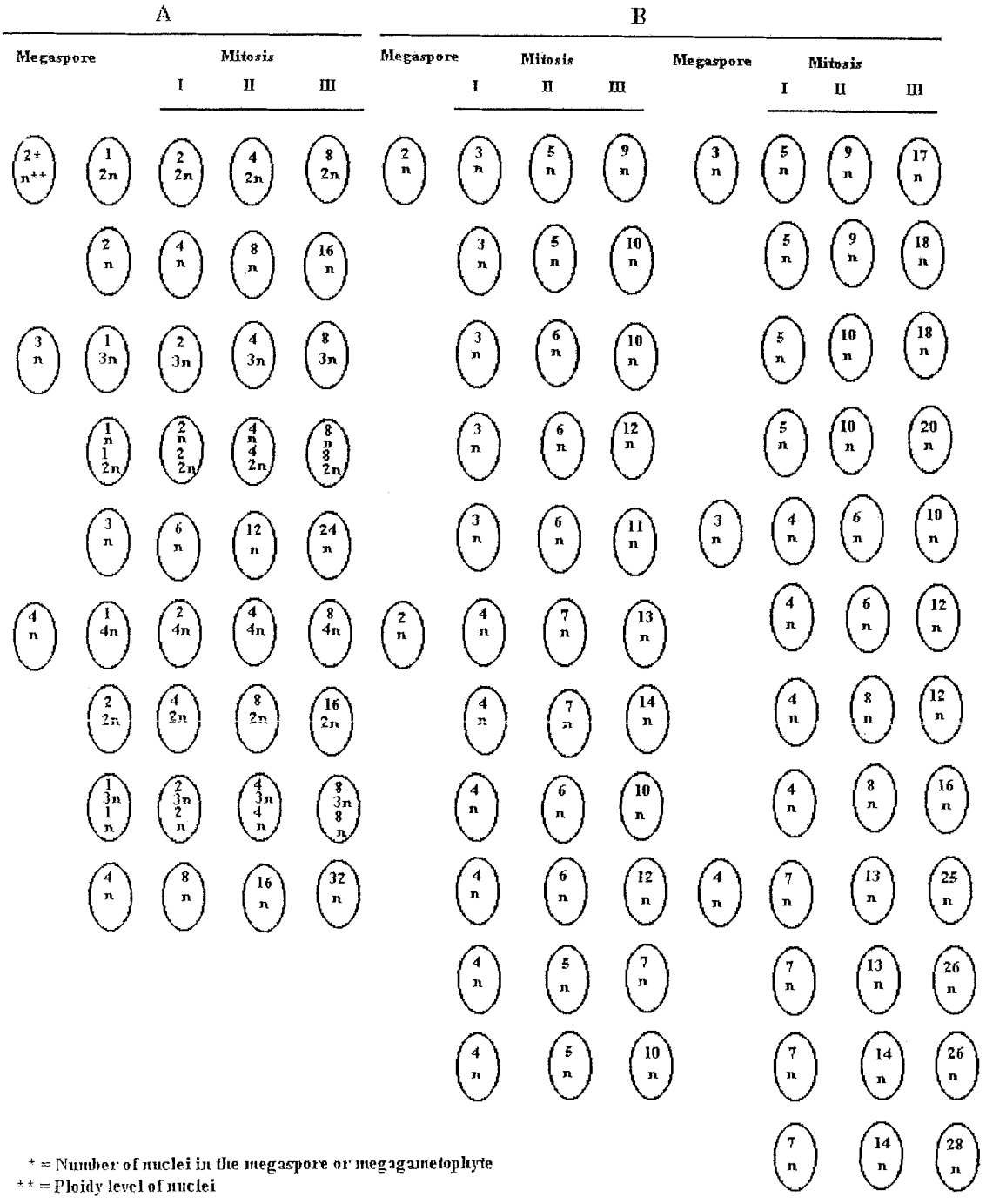


Fig. 4 Different types of coenocytic megaspore development in the *jp* clones: **A** different degrees of nuclear fusion; **B** lack of one or more mitotic divisions for one or more nuclei

tetranucleated macrospores were found only in clone H29 (Mariani et al. 1993). The five and six-nucleate MGs may account for the presence of mature MGs with 10 and 12 nuclei, assuming that the third mitotic division is suppressed. Therefore, the other MGs with an abnormal number of nuclei (Table 1) may be explained by the lack of one or more mitotic divisions for one or more nuclei of the coenocytic megaspores (Fig. 4). Macrogametophytes with more than 16 nuclei were not observed, even though they could be produced by multinucleated macrospores (Fig. 4). Therefore, it is reasonable to presume that a high number of nuclei is incompatible with normal functioning of the embryo sac and that not all the nuclei divide, or else some of them degenerate, in the coenocytic megaspore or MG. The fact that not all the macrospores develop into normally functioning MGs is supported by the observation of MGs at early developmental stages in mature ovules. The presence of a large vacuole may indicate an attempt by these MGs to develop, but their nuclei were somehow unable to divide, perhaps due to their uneven ploidy levels. Embryo sacs with polyploid nuclei may be disadvantaged compared to embryo sacs with normal haploid nuclei during their development.

The supernumerary nuclei in the micropylar end may act as supernumerary egg cells, even though their position and shape did not help in gaining an insight into their nature. Thus, an ultrastructural analysis is necessary to explain the nature of these supernumerary micropylar cells. In fact, the presence of a filiform apparatus, which is detectable in ultrathin sections, could discriminate between synergids and egg cells.

The analysis of the different stages of embryo-sac development revealed that *jp* mutants produce 2n MGs, deriving from 2n macrospores. In fact, 2n bi-, tetranucleated and mature MGs were identified on the basis of their nucleolar dimensions. The observed frequency of 2n eggs in all the *jp* mutants is significantly lower than that of binucleated macrospores previously determined by Mariani et al. (1993). This may be due to environmental effects but also to the fact that coenocytic megaspores may not all develop into embryo sacs; in fact degenerating MGs were found at all stages of development. Further, it is also possible that binucleated macrospores, in which fusion of nuclei does not occur, developed into eight-nucleate MGs after only two mitotic divisions, in which case bispory would have occurred and these MGs would have been counted as normals. However, assuming that each mature alfalfa ovary bears from eight to ten ovules, the frequency of 2n eggs, as determined by the mathematical system mentioned above, substantially confirms the seed-set data [(number of seeds produced/number of flowers pollinated) × 100] from 2x-4x crosses involving these mutants (Veronesi et al. 1990).

The presence of ovules with two MGs is consistent with the hypothesis that polyembryonic seeds are produced by the jumbo pollen mutants, and is further confirmed by the finding of one polyembryonic seed in their progenies. However, polyembryonic seeds may also derive from the fertilization of more than one egg cell, as shown by the presence, at the micropylar end, of supernumerary nuclei which could include more than one egg cell.

The abnormalities displayed in the macrogametogenesis of the *jp* mutants are of special interest for manipulating the ploidy level in alfalfa breeding programmes. In particular, due to their functional male sterility, the *jp* mutants are suitable for producing tetraploid progenies via bilateral sexual polyploidization. Research is currently in progress to verify the occurrence of polyembryony by the analysis of embryological development in the progeny of the jumbo pollen mutants.

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