Theor Appl Genet (1993) 85:873–881

THEORETICAL AND APPLIED GENETICS

Abnormal macrosporogenesis in five alfalfa (*Medicago sativa*) mutants producing 4n pollen*

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Received June 9, 1992; Accepted June 19, 1992
Communicated by F. Salamini

Summary. An analysis of micro- and macrosporogenesis in five diploid alfalfa mutants was carried out using a stain-clearing technique. All plants produced tetranucleated microspores and jumbo pollen due to the complete failure of the postmeiotic cytokinesis as well as bi- and trinucleated macrospores. The latter was due to the absence of cytokinesis after the first and second meiotic division of macrosporogenesis. Only one out of the five clones analyzed formed tetranucleated macrospores as a consequence of the total lack of cytokinesis after both meiotic divisions. The fusion of nuclei within binucleated macrospores resulted in 2n macrospores of the SDR type, recognizable on the basis of nucleolus dimension, confirming the ability of jumbo pollen (*jp*) mutants to produce 2n eggs at a high frequency. Nuclear fusion was also observed within tri- and tetranucleated macrospores. Although having the same genetic background, the five clones showed significant variability in the expression of abnormal cytokinesis during macrosporogenesis.

Key words: Reproductive mutants – Jumbo pollen – Alfalfa – Cytogenetics – Sexual polyploidization

Introduction

In the *Medicago sativa* complex a reproductive mutation is responsible for the production of 4n pollen, designated 'jumbo pollen' (McCoy and Smith 1983). The cause of this mutation is the failure of the postmeiotic cytokinesis during microsporogenesis (Pfeiffer and Bingham 1983). Jumbo pollen production is controlled by a single recessive gene, designated *jp*, which functions at both the diploid and tetraploid levels; homozygous recessive plants are completely self sterile (McCoy and Smith 1983). McCoy and Smith (1983) carried out an in-depth analysis of the cytology of 4n pollen production which showed that diploid sporophytes can produce tetraploid mature pollen grains with one vegetative and one generative nucleus. This was found to be a consequence of the fusion of the four nuclei in the tetranucleated microspores, even though a low frequency of pollen grains with an abnormal number of nuclei was also observed. However, these mutants behaved essentially as male steriles because the fertility of the 4n pollen produced by the diploid jp clones was very low; in fact only very few hexaploids (2n = 6x = 48) and octoploids (2n = 8x = 64) have been obtained in 4x-2x (*jpjp*) and 8x-2x (*jpjp*) crosses. No hybrid progeny has been obtained from tetraploid jp clones used as male parents, regardless of the ploidy level of the female parent (McCoy and Smith 1983). However, jumbo pollen plants have normal female fertility, and some of them produce an elevated frequency of 2n eggs. Moreover, at the tetraploid level this mutation produced a high frequency of dihaploids in 4x (*jpjpjpjp*)-2x crosses (McCoy and Smith 1983), and jumbo pollen plants have been effective in interspecific hybridization (McCoy and Smith 1984). In particular, these mutants are valuable because they are functionally male sterile and can, therefore, be used as 2n egg producers to obtain tetraploid progenies by unilateral and bilateral sexual polyploidization. Hence, the study of jumbo pollen mutants deserves particular attention for ploidy manipulation

* Research supported by National Research Council of Italy, special project RAISA, sub-project no. 2, paper no. 527
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and breeding purposes in cultivated alfalfa (*Medicago sativa* L., 2n = 4x = 32).

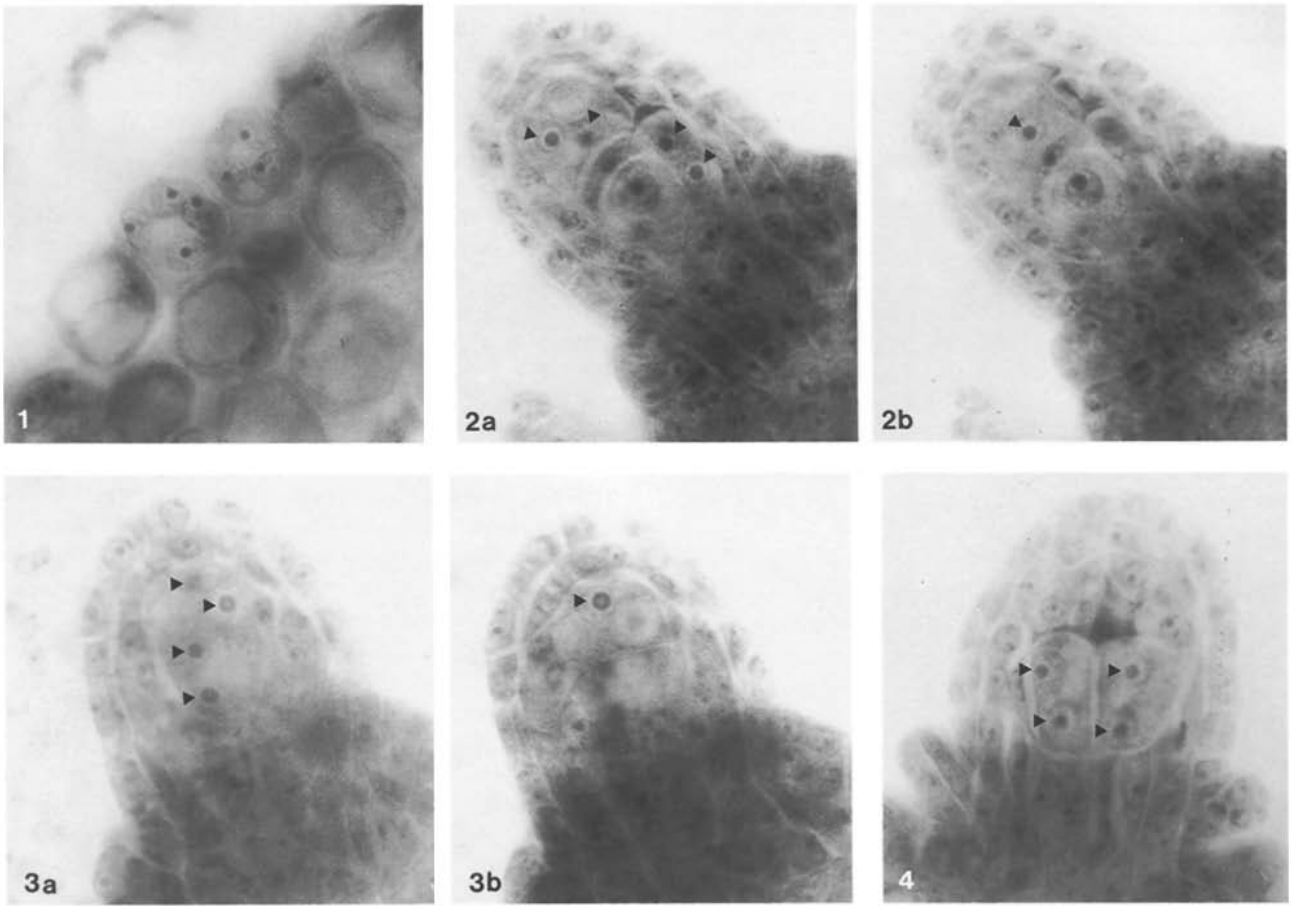
By means of a screening procedure based on pollen diameters that was carried out on a diploid experimental population of alfalfa, our research group has identified five jumbo pollen plants that are also characterized by the production of a high frequency of 2n eggs (Veronesi et al. 1990). The aim of the investigation presented here, was to analyze the micro- and macrosporogenesis of these plants from a cytological point of view in order to confirm their production of jumbo pollen and to determine the mechanism responsible for 2n egg production.

Materials and methods

The five jumbo pollen plants used in the present study (clones H21, H23, H25, H27, H29) were selected from an F_2 obtained from the cross of the diploid clone HY6 with diploid plants of CADL (Pfeiffer and Bingham 1983). These jp plants are characterized by their production of pollen grains that could be classified as jumbo pollen grains on the basis of their diameters, as indicated by McCoy and Smith (1983). Small buds of the jp clones were fixed in FAA, and the cytological analysis was performed using a stain-clearing technique (Stelly et al. 1984), which in alfalfa has proven to be effective for observing the stages of both micro- and macrosporogenesis (Tavoletti et al. 1991). A sample of ovules, ranging from 83 (clone H25) to 117 (clone H21) per plant, was analyzed; in total, 468 ovules were examined. The chi-square test was applied to verify differences in the production of normal and abnormal macrosporogenesis among the five jp clones analyzed (Steel and Torrie 1980).

Results

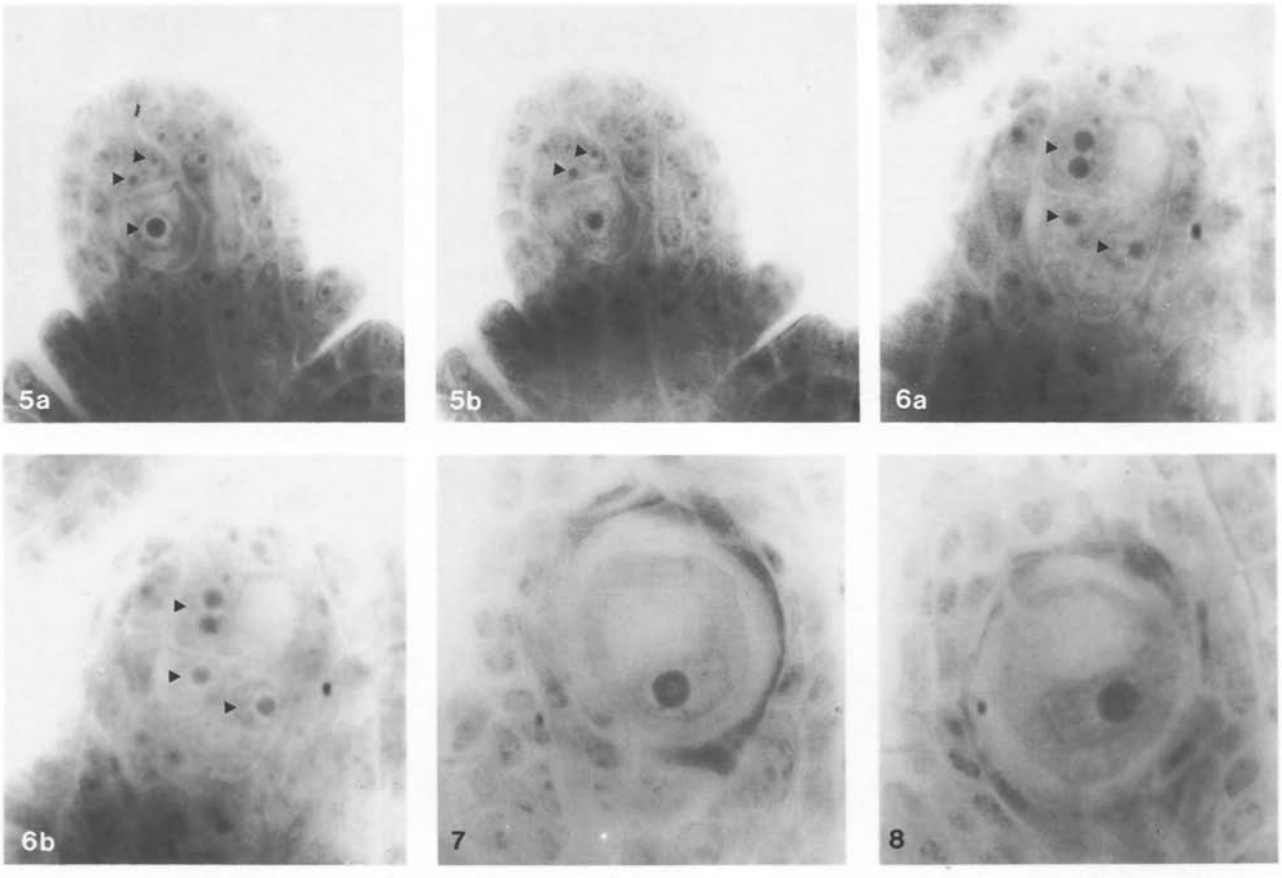
Microsporogenesis analysis confirmed that all five clones are jumbo pollen mutants; in fact they produced only tetranucleated microspores due to the failure of postmeiotic cytokinesis (Fig. 1). These plants also showed abnormalities in cytokinesis during macro-



Figs. 1–4. 1. Tetranucleated microspores. × 2100. **2a, b.** Triads with one binucleated macrospore at the micropilar (on the *left*) and at the chalazal end (on the *right*). (Two planes of focus, × 2100). **3a, b.** Dyad with two binucleated macrospores. × 2100. **4.** Two chalazal binucleated macrospores. × 2100

Table 1. Results of the cytological analysis of macrosporogenesis in the five jumbo pollen clones: total number of ovules analyzed and percentage of normal and abnormal macrosporogenesis observed

jp clones	Total ovules	Normal tetrads	Binucleated macrospores			Trinucleated macrospores		Tetranucleated macrospores	Partial walls	Aborted ovules
			Triads		Dyads	Dyads				
		a	b	c	d	e	f	g	h	i
H21	117	58.12	13.67	8.55	6.84	0.85	2.56	0.00	0.85	8.56
H23	88	56.82	5.68	3.41	1.14	0.00	1.13	0.00	0.00	32.23
H25	83	48.19	14.46	3.61	7.23	6.02	8.43	0.00	2.41	9.64
H27	89	48.31	12.36	2.25	13.48	0.00	3.37	0.00	2.25	17.98
H29	91	24.18	13.19	2.20	25.27	2.20	7.69	9.89	6.59	8.79

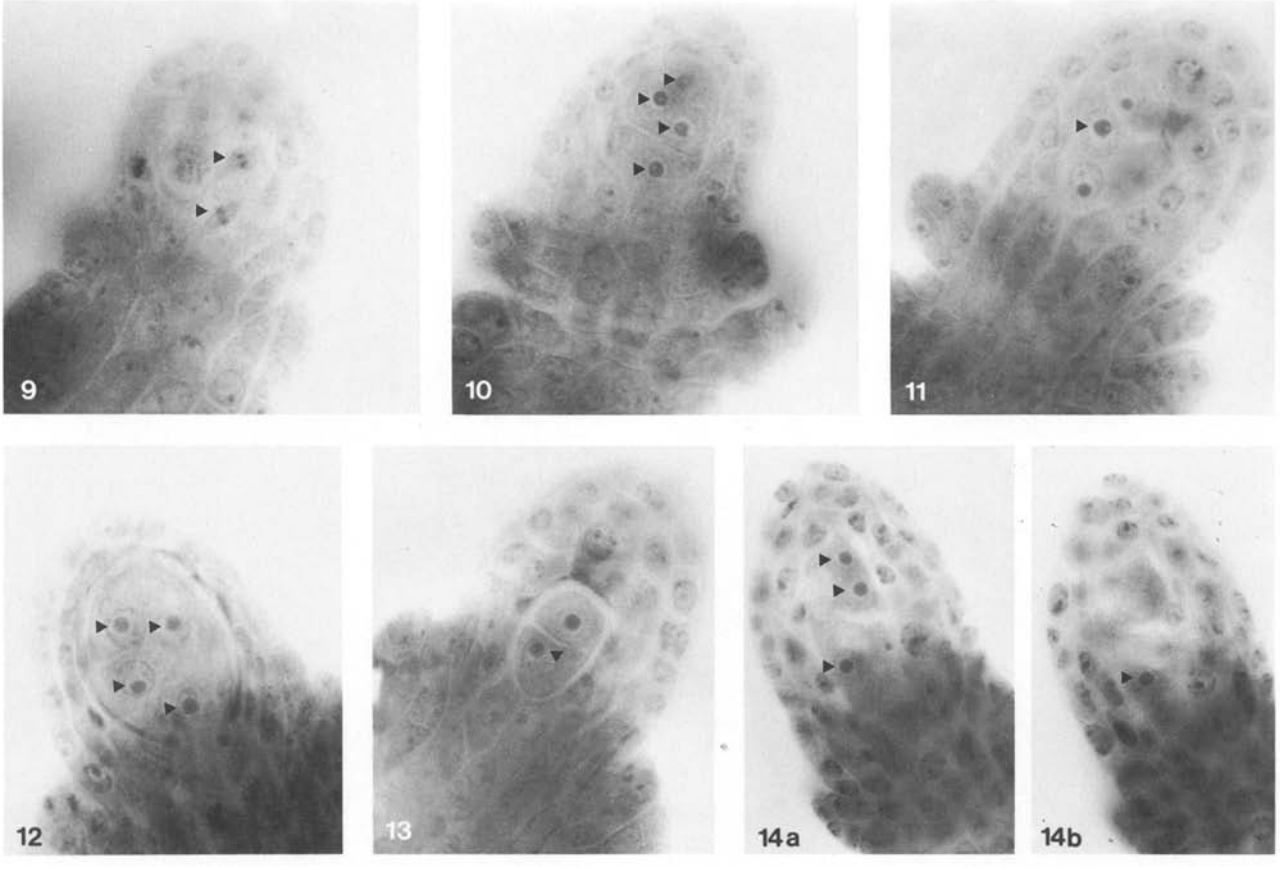


Figs. 5–8. 5a, b. Dyad with one 2n chalazal macrospore and one binucleated micropilar macrospore. (Two planes of focus, × 2100). **6a, b.** Dyad with one 2n micropilar macrospore and one binucleated chalazal macrospore. (Two planes of focus, × 2500). **7.** Functional n macrospore. × 2500. **8.** Functional 2n macrospore. × 2500

sporogenesis. In alfalfa normal macrosporogenesis leads to the production of linear tetrads of macrospores: the three micropilar macrospores degenerate and the chalazal one becomes the functional macrospore, giving rise to the female gametophyte. In the five jumbo pollen clones analyzed, normal tetrads of the macrospores as well as dyads and triads, the latter resulting from the absence of cytokinesis after the first and/or the second meiotic division, were observed.

The frequencies of normal and abnormal macrospore arrangements, together with the frequencies of aborted ovules, at the sporad stage are reported in Table 1 for the five clones. Normal cytokinesis after the first division followed by the absence of cytokinesis after the second division at the chalazal, the micropilar or at both poles (Table 1b–d) resulted in the production of binucleated macrospores. Figure 2a and b shows two triads with one binucleated macrospore at either the chalazal pole or the micropilar pole in the same ovule; Fig. 3a and b shows a dyad with two binucleated macrospores; Fig. 4 shows an ovule with two chalazal binucleated macrospores. The fusion of the two nuclei in the binucleated macrospores resulted in 2n macrospores (Figs. 5a and b, 6a and b) that, when functional, give rise to unreduced embryo sacs of the second division restitution (SDR) type. On the basis of nucleolus dimension it was possible to discriminate between n and 2n functional macrospores (Figs. 7 and 8). The lack of cytokinesis after the first division (Fig. 9), followed by the lack of cytokinesis after the second division at the micropilar or chalazal end (Fig. 10), resulted in dyads with one single-nucleated and one trinucleated macrospore (Table 1e and f). The fusion of two of the three nuclei within a trinucleated macrospore can occur, as shown in Fig. 11.

The total absence of cytokinesis after both meiotic divisions, which consequently resulted in tetranucleated macrospores, was observed only in clone H29 (Table 1g and Fig. 12). Abnormal cytokinesis also determined the production of partial walls, as shown



Figs. 9–14. 9. M-II. Lack of cytokinesis after the first division. × 2100. **10.** Dyad with one trinucleated micropilar macrospore. × 2100. **11.** Fusion of two nuclei in a trinucleated chalazal macrospore. × 2100. **12.** Tetranucleated macrospore of clone H29. × 2100. **13.** Partial wall due to abnormal cytokinesis after the second division at the chalazal end. × 2100. **14a, b.** Partial wall produced after the first division followed by the total lack of cytokinesis after the second division. × 2100

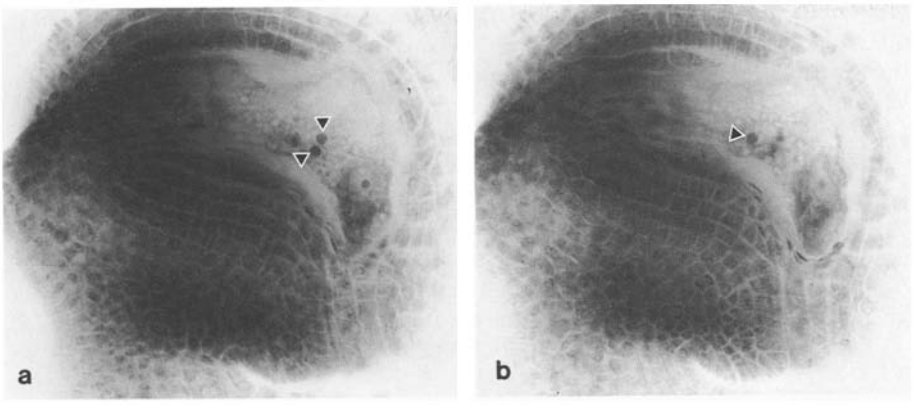


Fig. 15a, b. Abnormal embryo sac with three polar nuclei. × 1050

Table 2. Results of the chi-square tests applied to verify homogeneity among the five jp clones for the different classes of ovules

a) Contingency table showing observed values (expressed as percentages in parentheses) of the different classes of ovules for each of the jp clones analyzed

jp clones	Total	Classes of ovules			
		A	B	C	D
H21	117	68 (58.12)	34 (29.06)	5 (4.27)	10 (8.55)
H23	88	50 (56.82)	9 (10.23)	1 (1.13)	28 (31.82)
H25	83	40 (48.19)	22 (26.51)	13 (15.66)	8 (9.64)
H27	89	43 (48.31)	27 (30.34)	3 (3.37)	16 (17.98)
H29	91	22 (24.17)	37 (40.67)	24 (26.37)	8 (8.79)

Chi-square = 97.26 (12 df), significant at $P < 0.005$

b) Results of chi-square tests calculated to compare each jp clone with all the others (each chi-square has 3 degrees of freedom)

	H23	H25	H27	H29
H21	24.88 $P < 0.005$	8.06 $0.025 < P < 0.05$	4.60 $0.10 < P < 0.25$	33.58 $P < 0.005$
H23		27.83 $P < 0.005$	13.79 $P < 0.005$	60.16 $P < 0.005$
H25			9.34 $0.025 < P < 0.05$	11.97 $0.005 < P < 0.01$
H27				27.32 $P < 0.005$

in Figs. 13 and 14a and b; in Table 1h the frequency of macrosporogenesis with partial walls for each of the five mutants is shown. All of these plants showed a certain number of aborted ovules (Table 1i).

Preliminary results on clone H25 indicated that these mutants can also produce embryo sacs with supernumerary nuclei; Fig. 15a and b shows an embryo sac with three polar nuclei. The chi-square test was applied to verify if the five jp clones were homogeneous for the abnormalities in cytokinesis observed during megasporogenesis. For this purpose, the classes from 'a' to 'i' of Table 1 were grouped, and a contingency table (Table 2a) with the following four classes (A, B, C, D) of ovules was obtained:

A) ovules with tetrads of reduced macrospores produced by normal macrosporogenesis: class 'a';
B) ovules with binucleated macrospores produced by a normal cytokinesis after the first division followed by an abnormal cytokinesis after the second division: sum of classes 'b', 'c', 'd' and ovules of class 'h' with partial second division walls (two ovules of clone H27 and one ovule of clone H25);
C) ovules showing abnormal cytokinesis after both

the first and second division resulting in the formation of tri- and tetranucleated macrospores: sum of classes 'e', 'f', 'g' and ovules of class 'h' not included in class B (one ovule for each of clones H21 and H25 and six ovules of clone H29);
D) aborted ovules: class 'i'.

The chi-square calculated for this contingency table with $r = 5$ rows (the five jp clones) and $c = 4$ columns (the four classes of ovules) had $(r-1)(c-1) = 12$ degrees of freedom, and the results were highly significant, indicating the presence of differences among the five jp clones (Table 2a). In order to verify the possibility of pooling data of some clones because of homogeneity, each jp clone was compared with all the others. The results obtained (Table 2b) indicated that only clones H21 and H27 were homogeneous and, therefore, data were pooled for the subsequent analysis.

In order to determine the most interesting differences among clones, classes of ovules of Table 2a were grouped as follows in order to obtain three two-cell tables:

1) non-aborted ovules (A + B + C) and aborted ovules (D), (Table 3);
2) normal macrosporogenesis (A) and abnormal macrosporogenesis (B + C) within non-aborted ovules (Table 4);
3) macrosporogenesis with binucleated macrospore production (B) and macrosporogenesis with tri- and tetranucleated macrospore production (C) within abnormal macrosporogenesis (Table 5).

The chi-squares calculated for these three two-cell tables, each with 3 *df*, were highly significant (see Tables 3a, 4a and 5a), and consequently comparisons within each two-cell table were performed.

The high heterogeneity among clones with respect to the number of aborted ovules produced (Table 3a) was essentially due to the behavior of clone H23. In fact, clones H21, H25, H27 and H29 were homogeneous for frequency of non-aborted and aborted ovules, as confirmed by a non-significant chi-square (Table 3b). Therefore, data from these clones were pooled, and a comparison with clone H23 was carried out (Table 3c). The highly significant chi-square that was obtained indicated that clone H23 exhibited a lower frequency of non-aborted ovules (68.18%) than the other four clones (88.95%). Therefore, the five jp clones were classified as shown in Table 3d.

The chi-square test also revealed the presence of highly significant differences among clones with regard to the relative amount of normal and abnormal macrosporogenesis within non-aborted ovules (Table 4a). One group of homogeneous jp clones was identified as consisting of clones H25, H21 and H27 that were

Table 3. Tests of homogeneity among the jp clones for the production of non-aborted and aborted ovules (expected values are not shown)

	Non-aborted (A+B+C)		Aborted (D)		Chi-square	P[d]
	Observed	%[c]	Observed	%[c]		
Comparisons						
a)						
H23	60	68.18	28	31.82		
H25	75	90.36	8	9.64	25.12	$P<0.005$
(H21+H27)[a]	180	87.38	26	12.62	(3 *df*)	
H29	83	91.21	8	8.79		
b)						
H25	75	90.36	8	9.64		
(H21+H27)[a]	180	87.38	26	12.62	1.16	$0.50<P<0.75$
H29	83	91.21	8	8.79	(2 *df*)	
c)						
H23	60	68.18	28	31.82		
(H21+H25+ H27+H29)[a]	338	88.95	42	11.05	24.23 (1 *df*)	$P<0.005$
d) Summarized results[b]: H23 < H21 = H25 = H27 = H29						

[a] Pooled data for these clones
[b] Ordered on the basis of the number of non-aborted ovules produced
[c] Observed value/total number of ovules produced
[d] Probability values (P) based on chi-square

Table 4. Tests of homogeneity among the jp clones for the production of normal and abnormal macrosporogenesis within non-aborted ovules (expected values are not shown)

	Normal (A)		Abnormal (B+C)		Chi-square	P^d
	Observed	%[c]	Observed	%[c]		
Comparisons						
a)						
H23	50	83.33	10	16.67		
H25	40	53.33	35	46.67	50.07	$P<0.005$
(H21+H27)[a]	111	61.67	69	38.33	(3 *df*)	
H29	22	26.51	61	73.49		
b)						
H25	40	53.33	35	46.67	1.52	$0.1<P<0.25$
(H21+H27)[a]	111	61.67	69	38.33	(1 *df*)	
c)						
H23	50	83.33	10	16.67	12.26	$P<0.005$
(H21+H25+H27)[a]	151	59.22	104	40.78	(1 *df*)	
d)						
H29	22	26.51	61	73.49	26.81	$P<0.005$
(H21+H25+H27)[a]	151	59.22	104	40.78	(1 *df*)	
e)						
H23	50	83.33	10	16.67	44.99	$P<0.005$
H29	22	26.51	61	73.49	(1 *df*)	
f) Summarized results[b]: H23 < H21 = H25 = H27 < H29						

[a] Pooled data for these clones
[b] Ordered on the basis of the frequency of abnormal macrosporogenesis
[c] Probability values (*P*) based on chi-square
[d] (Observed values/total number of non-aborted ovules)·100

Table 5. Test of homogeneity among the jp clones for the production of different classes of abnormal macrosporogenesis: binucleated macrospores (Class B) and tri- and tetranucleated macrospores (Class C) (expected values are not shown)

	Class B		Class C		Chi-square	P^d
	Observed	%[c]	Observed	%[c]		
Comparisons						
a)						
H23	9	90.00	1	10.00		
H25	22	62.86	13	37.14	16.55	$P<0.005$
(H21+H27)[a]	61	88.41	8	11.59	(3 *df*)	
H29	37	60.66	24	39.34		
b)						
H23	9	90.00	1	10.00	0.022	$0.75<P<0.90$
(H21+H27)[a]	61	88.41	8	11.59	(1 *df*)	
c)						
H25	22	62.86	13	37.14	0.045	$0.75<P<0.90$
H29	37	60.66	24	39.34	(1 *df*)	
d)						
(H23+H21+H27)[a]	70	88.61	9	11.39	16.48	$P<0.005$
(H25+H29)[a]	59	61.46	37	38.54	(1 *df*)	
e) Summarized results[b]: H21 = H23 = H27 > H25 = H29						

[a] Pooled data for these clones
[b] Ordered on the basis of the frequency of abnormal macrosporogenesis of class B
[c] Probability values (*P*) based on chi-square
[d] (Observed values/total number of ovules with abnormal macrosporogenesis)·100

characterized by a percentage of normal tetrads within non-aborted ovules equal to 53.33% for clone H25 and 61.67% for the pooled H21-H27 clones. Data from these clones were pooled because of the non-significant chi-square obtained, as reported in Table 4b. Clones H23 and H29 showed quite a different and opposite behavior compared to the other jp clones (Table 4a) due to the prevalence of normal macrosporogenesis in clone H23 (83.33%) and the prevalence of abnormal macrosporogenesis in clone H29 (73.49%). Both comparisons between each of these two clones and the pooled H21-H25-H27 clones were highly significant (Table 4c and d); clones H23 and H29 also differed significantly between each other (Table 4e). Therefore, on the basis of the frequency of abnormal macrosporogenesis within non-aborted ovules, the five jp clones analyzed can be classified as shown in Table 4f.

Differences among clones were also found during abnormal macrosporogenesis at the time the abnormalities were expressed in cytokinesis (Table 5a). In fact, clones H21, H23 and H27 showed a predominance of abnormal cytokinesis after the second division that resulted in the prevailing formation of binucleated macrospores. The test of homogeneity for clones H21, H23 and H27 is reported in Table 5b. An increased frequency in abnormal cytokinesis after the first meiotic division was shown by clones H25 and H29. In fact, the percentage of ovules belonging to class C increased in these two clones (37.14% and 39.34% for H25 and H29, respectively) when compared to the relative frequencies of clones H21, H23 and H27 (Table 5a). The test of homogeneity for clones H25 and H29 is reported in Table 5c. It is interesting to note that very low chi-square values were obtained for comparisons b and c of Table 5, indicating a strong similarity within each of the two groups of clones. These two groups of clones ('H21, H23, H27' and 'H25, H29') differed significantly between each other (Table 5d). Therefore, these results indicate that, even though all five jp clones showed a prevailing production of binucleated versus tri- and tetranucleated macrospores within abnormal macrosporogenesis, clones H25 and H29 showed a significant increase in the frequency of abnormal cytokinesis after the first division in comparison with the group of clones H21, H23 and H27 (Table 5).

Discussion

Mutations leading to the production of tetranucleated microspores, due to the lack of both reductional and equational cytokinesis during microsporogenesis have also been found in *Melilotus alba* (Castetter 1925), *Pisum sativum* (Gottschalk and Kaul 1974), *Glycine max* (Albertsen and Palmer 1979; Cutter and Bingham 1977), *Zea mays* (Beadle 1931), *Cerasus avium* (Mashkina 1979) and *Brassica japonica* (Heyn 1977).

The results of the investigation presented here together with those of McCoy and Smith (1983), show that the behavior of alfalfa jumbo pollen mutants resembles very closely that of soybean plants homozygous recessive at the ms_1 locus. In fact, the alfalfa mutants are functionally male sterile due to the production of coenocytic microspores through the same mechanism shown in soybean, i.e. the failure of cytokinesis after telophase II. Moreover, in soybean the ms_1 system has a pleiotropic effect on female development in that ms_1ms_1 plants exhibit a high frequency of polyembryony, haploidy and polyploidy (Beversdorf and Bingham 1977). Cutter and Bingham (1977) found that this characteristic was related to the production of abnormal embryo sacs with supernumerary nuclei in the regions of the secondary nucleus and egg apparatus. Kennel and Horner (1985) observed that in ms_1 mutants of soybean, megagametophytes with more than eight nuclei were produced as a result of the formation of coenomegaspores. Megagametophytes with up to four eggs and also displaying a high frequency of aborted ovules were observed, which explains the characteristic polyembriony and reduced female fertility of these soybean mutants. Our results show that jumbo pollen alfalfa plants were also characterized by abnormalities in macrosporogenesis that resulted in the absence of cytokinesis after the first and/or second meiotic division. Consequently, functional bi- and trinucleated macrospores were produced. Tetranucleated macrospores were produced only by clone H29 and resulted from the total absence of cytokinesis after both meiotic divisions. 2n SDR functional macrospores resulted from the fusion of nuclei within the binucleated macrospores. This mechanism is the same as that described by Pfeiffer and Bingham (1983) in clone HY6; however, abnormalities in cytokinesis during macrosporogenesis were more pronounced in the 5 jp clones as shown by the presence of tri- and tetranucleated functional macrospores. This is also confirmed by the presence of partial walls, a result of incomplete cytokinesis and aborted ovules; this is also found in soybean ms_1 mutants. Moreover, clone H25 produced mature embryo sacs with supernumerary nuclei. Research is in progress to study the development of multinucleated macrospores in order to verify if the production of embryo sacs with supernumerary nuclei in alfalfa jp mutants is due to the formation of coenocytic functional macrospores.

It is interesting that the five related jp clones had a variable expression of abnormal cytokinesis during macrosporogenesis. In particular, clone H23 was characterized by the highest number of aborted ovules and the lowest frequency of abnormal macrosporo-

genesis within the non-aborted ovules. This could indicate that in clone H23, abnormal macrosporogenesis led to ovules that were prevalently incapable of developing female gametophytes. On the contrary, the remaining four jp clones produced a higher frequency of abnormal macrospores that retained the capacity to develop. They had a reduced frequency of aborted ovules and an increased frequency of abnormal macrosporogenesis when compared with clone H23. Clone H29 also differed significantly from all of the other jp clones because it had the highest frequency of abnormal macrosporogenesis within non-aborted ovules. It is noteworthy that only this clone produced tetranucleated macrospores. Therefore, H29 deserves particular attention for further analysis because of its high expression of abnormalities during macrosporogenesis.

Two types of mechanisms for 2n gamete formation have been defined by Peloquin (1983) who underlined, working on diploid *Solanum* spp., the higher level of heterozygosity transmitted by FDR compared to SDR. However, the usefulness of both FDR × FDR and FDR × SDR matings for maximizing heterosis in autotetraploids has been stressed by Watanabe et al. (1991). Therefore, these findings confirm that all five jp mutants could be useful, because of their functional male-sterility, in obtaining interspecific hybrids and, due to the high frequency of 2n eggs, for producing tetraploid progenies by sexual polyploidization.

Acknowledgements. The authors wish to thank Prof. E. T. Bingham, Agronomy Department, University of Wisconsin, Madison, for critical reading of the manuscript. Thanks are also due to Sister Mary Traynor, Ph.D., University of Perugia, Italy, for her help with the English of the manuscript and to Mr. A. Bolletta for his technical assistance in preparing photographs.

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