Theor Appl Genet (1984) 67:439–442

Wheat microspore embryogenesis during in vitro anther culture

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Received May 3, 1983; Accepted November 20, 1983
Communicated by G. Wenzel

Summary. Cytological analysis of microspore embryogenesis during in vitro culture reveals a high mortality in the first week and a latency phase of about one week before the first embryogenic mitosis. Genotypic differences observed during our wheat anther culture do not seem to originate at the induction level but are linked to the different abortion rates.

Key words: Wheat – Multicellular pollen grains – Embryos – Cytology

Introduction

Green plants having a microspore origin can be obtained by the in vitro culture of wheat (*Triticum aestivum*) anthers. For androgenesis, microspores are harvested at a stage close to the first microspore mitosis. They can produce embryos after a one month culture on an appropriate solid agar media. However, there is very little quantitative data concerning these embryos' formation and evolution. The existence of a latency phase before initiation of the embryonic process in the microspores seems to be confirmed by Pelletier's observation in tobacco (1979) and by those of Hesky-Mesch (1976) and Pan and Gao (1980) in wheat. The cytological analysis conducted by the latter authors reveals the presence of numerous multicellular pollen grains (MPG) in wheat anthers after two weeks in culture.

Taking into account these data, the present study deals with microspore embryogenesis after one week of in vitro culture. Variations in MPG and young embryo frequencies were followed for 10 days. Two genotypes with different androgenetic yields were compared in order to define the origin of the genotypic differences observed in anther cultures.

Materials and methods

Two genotypes, A (F_4) and B (F_8), were chosen as the basic material for a cytological study because of their very different androgenetic aptitudes in vitro (Table 1): B was a more responsive genotype while A was a less responsive one. The anther donor plants were cultivated in a greenhouse during April and May. Sixteen A genotype and twenty-four B genotype spikes were harvested. After a one-week cold treatment (3 °C) the spikes were sterilized and anther culture was initiated. Three anthers taken from the medial area of each spike were fixed in acetic alcohol (1 : 3) and used to determine the microspore stage at the time of culture initiation. The culture medium contained potato extract (Henry-De Buyser 1981) with $2 \cdot 10^{-4}$ M iron EDTA and was solidified by 5.5 g/l agarose. Anthers were cultured at 28 °C ± 2 ° with a 16 h photoperiod.

After 7, 9, 11, 13 and 17 days of culture, 6 anthers were removed from each A or B spike and fixed in acetic alcohol (1 : 3). Microspores were considered to be alive when their nuclei and cytoplasm stained well in acetic carmine. A sampling of 200 microspores taken from each anther or from a mixture of 3 anthers were observed in order to determine the developmental stage.

To confirm results obtained from A and B, 25 other genotypes were used. Six spikes per genotype were collected and anthers were plated on the same culture medium. Anthers were only taken after 13 days of culture.

Results

The results obtained on the unharvested anthers confirm the genotypic differences observed previously (Table 1). No embryos are produced by the A genotype while the B genotype has at least one embryogenic anther in nearly half the cultivated spikes.

Table 1. Androgenetic yields of two wheat genotypes

Genotypes	A	B	A[a]	B[a]
No. of cultivated anthers	8,760	15,300	1,179	1,917
No. of remaining anthers	8,760	15,300	651	1,125
No. of embryos obtained	31	1,280	0	29
%	0.4	8.4	0	2.6
No. of regenerated green plants	1	232	–	–
%	0.01	1.51	–	–
Frequency of embryogenic spikes	19/146	139/255	0/16	10/24
Maximum no. of embryogenic anthers per spike	5	27	0	4
Maximum no. of embryos per embryogenic anther	3	21	–	5

[a] Androgenetic yield during the experiment

Table 2. Evolution of cultivated anthers and microspores

Observation	Genotype	Culture age (days)					
		0	7	9	11	13	17
Percentage of anthers containing living microspores (%)	A	100	94.5	26	15.8	17	2.1
	B	100	100	45.5	42.4	31.6	17.6
Microspores survival rate (%)	A	98.94	10.69	1.79	2.24	1.40	0.05
	B	98.98	26.26	4.71	2.63	1.97	0.70
Average no. of nuclei per surviving microspore	A	1	1.7	2.4	4	8.8	20.1
	B	1	1.5	2	4	6.5	9.1
Maximum no. of nuclei per microspore	A	1	7	12	20	32	80
	B	1	12	20	25	55	100

The cytological analysis of the anthers harvested before culture initiation shows that all microspores possessed one nucleus preceding the first microspore mitosis. At this stage only 1% of the microspores are dead in both genotypes and no microspore dimorphism is observed. Thus, there are no visible differences between the A and B genotypes at the time of culture initiation.

Microspore survival rate

After 7 days of culture practically all the anthers still contain living microspores (Table 2). Mortality increases between day 7 and 9. At this stage the two genotypes show a difference: 75% of the A genotype anthers no longer have any living microspores against only 50% for B. After 17 days of culture the microspore survival rate becomes very low.

Microspore evolution in vitro

For almost every anther harvest date the average number of nuclei per microspore is higher in the A than in the B genotype. Mitosis in the former genotype starts earlier. Thus for A only MPG with more than 6 nuclei remain after 17 days culture while B still has numerous bi- or uninucleated microspores or MPG with 3 to 5 nuclei. Moreover, for every harvest date, the most advanced MPG were observed in the B genotype.

The choice of spikes was correct since they all have uninucleated microspores when the culture is initiated. Thus, the microspore stage alone is inadequate for use in predicting which spikes are efficient since only 25% spikes undergo embryogenesis.

Cytological observation of the microspores after 7 days of culture (Table 3) shows that a large number of surviving microspores are uni- or binucleated. A latency phase is observed, as in tobacco (Pelletier 1979), before the embryonic development begins. Two days later, development continues and the number of MPG having more than 5 nuclei increases. The maximum number of nuclei observed is 12 for A and 20 for B.

After 11 days of culture a further increase in the frequency of MPG having 3 to 5 nuclei is observed (Table 3). This seems to indicate the existence of a second division induction phase between 9 and 11 days of culture, from which the MPG, having more than 10

Table 3. Microspore evolution during culture

Culture age (days)	Genotype	Data	Total effectives observed	State of microspore evolution (no. of nuclei)							
				Dead	1	2	3 to 5	6 to 10	11 to 20	21 to 50	more than 50
0	A	E[a]	3,100	33	3,067						
		%	100	1.06	98.94						
	B	E	4,500	46	4,454						
		%	100	1.02	98.98						
7	A	E	6,400	5,716	321	274	87	2			
		%	100	89.31	5.02	4.28	1.36	0.03			
	B	E	9,500	7,006	1,630	636	224	4			
		%	100	73.75	17.16	6.69	2.36	0.04			
9	A	E	19,200	18,855	110	136	82	13	4		
		%	100	98.20	0.57	0.71	0.43	0.07	0.02		
	B	E	26,400	25,158	690	338	138	65	11		
		%	100	95.29	2.61	1.28	0.52	0.25	0.04		
11	A	E	18,800	18,378	121	83	104	97	15	2	
		%	100	97.75	0.67	0.44	0.55	0.51	0.08	0.01	
	B	E	25,000	24,343	152	125	187	138	53	2	
		%	100	97.37	0.61	0.50	0.75	0.55	0.21	0.01	
13	A	E	18,800	18,537	6	21	55	104	69	8	
		%	100	98.60	0.03	0.11	0.29	0.55	0.37	0.04	
	B	E	27,200	26,664	47	109	130	158	83	8	1
		%	100	98.03	0.17	0.40	0.48	0.58	0.31	0.03	0.004
17	A	E	19,200	19,191	–	1	–	3	3	1	1
		%	100	99.953	–	0.005	–	0.016	0.016	0.005	0.005
	B	E	26,200	26,015	30	13	35	61	33	11	2
		%	100	99.29	0.11	0.05	0.13	0.23	0.13	0.04	0.01

[a] E = effective

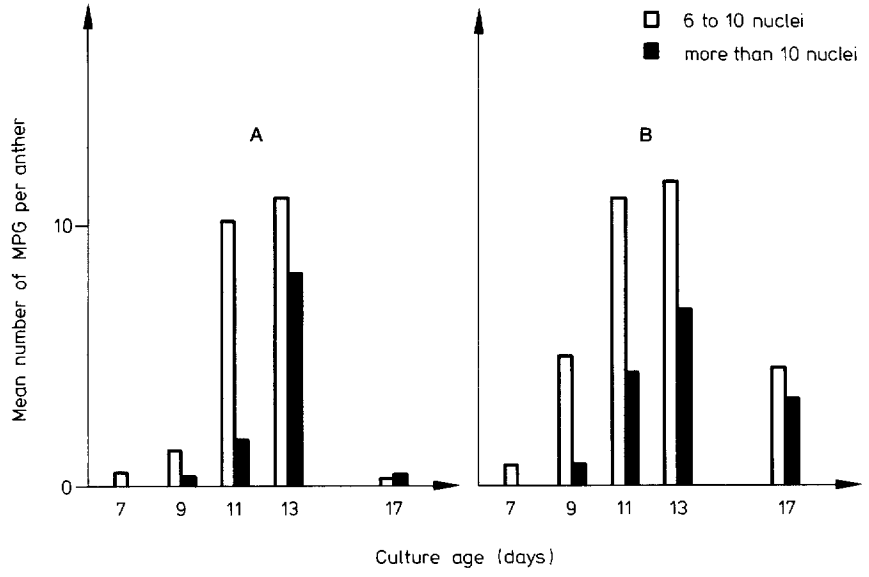

Fig. 1. MPG evolution

nuclei and whose frequency increases sharply between 11 and 13 days of culture, could originate.

Cytological analysis carried out 2 days later indicate that MPG degeneration has begun. Therefore, there is a critical phase between 11 and 13 days of culture. This stage could correspond with the rupture of the MPG exine. After day 13, there are 19.3 MPG with more than 5 nuclei per average anther for A, 18.4 for B and 21.4 for the 25 other tested genotypes. This shows that the induction rate is quite similar for all genotypes. Several exceptional anthers can be observed in all genotypes, corresponding to an embryogenetic initiation in 25% of the microspores (for example 177 MPG from 696 microspores).

From the 13th day on, degeneration of young embryos intensifies (Fig. 1) and only a fraction continue their development. At day 17 the survival rate for the A genotype is very low and its difference with the B genotype become obvious. The latter has a larger number of well-structured embryos. The most important observation during this period concerns the high degeneration rate: of the MPG observed on day 13, only a few are able to produce true embryos.

Discussion

The cytological analysis of microspore embryogenesis during in vitro anther culture reveal several interesting points:

– the first concerns the high microspore mortality observed during the first week of culture.

– the second point concerns induction of the embryonic divisions. Under our experimental conditions, wheat has a latency phase of about one week before the first embryonic divisions. It would seem that the earliest divisions are not the source of the embryos obtained by anther culture but instead provide MPG which rapidly degenerate. During this culture phase, the microspores undergo extensive transformations (Bhojwani et al. 1973; Raquin et al. 1982).

Furthermore, the induction rate can be very high in certain anthers. This supports the hypothesis that in wheat androgenesis induction is not a limiting factor since its frequency is high (1.5% of the microspores) in all tested genotypes. This fact seems to indicate that the genotypic effects observed in wheat anther cultures do not originate at the androgenesis induction level. The essential objective consists in increasing the MPG survival rate so that they can progress towards more advanced embryonic stages while maintaining a good structure.

– the third point is linked to the differences between the two genotypes A and B, essentially their different degeneration rates from the 13th day on. This implies that research should be narrowed on the in vitro culture between days 7 and 13 in order to insure the induced embryos development. The reason why the A genotype embryos degenerate more frequently than the B embryos should also be studied. If this degeneration depends on anther diploid tissues, this should explain the sporophytic determination of the embryo ratio (Raquin 1982). Chromosomal abnormalities have also been observed, they could be the cause of some abortions.

Genotypic effects observed during wheat anther culture can be related to the different genotypic abortion rates but not to the androgenetic induction rate which, on the average, exceeds 25 microspores per anther in the 27 tested genotypes. The analysis also demonstrates the potential of this technique: for the B genotype an average of more than 3 MPG with more than 10 nuclei per anther can be observed on the 17th day culture. If this observation is extrapolated to an average cultured spike, the result would be about 250 embryos. In fact, for control anthers, only one embryo per spike and 12 for the best spike, are obtained after 4 to 6 weeks of culture.

Another frequently debated point concerns the type of nuclei participating in the embryos formation. No vegetative or reproductive nuclei have been observed in an MPG: the only structures that can be seen during the first phase of culture are more or less intensely stained nuclei (Pan and Gao 1980; Zeng and Ouyang 1980). The young embryos' nuclei stain uniformly and the embryos seem to originate in the vegetative cell.

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