Microspore development during *in vitro* androgenesis in triticale

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Abstract

Microspore division was monitored in three triticale (× *Triticosecale* Wittmack) genotypes over 21 d of *in vitro* anther culture, on two media differing in their 2,4-dichlorophenoxyacetic acid content. After low temperature (4 ºC) pre-treatment for two weeks, all the microspores were still alive, but they began to die from day one of culture. Both genotype and culture medium affected the number of microspores that aborted over time (82 - 97 % by day 21), the number of microspores that underwent the first symmetrical division (> 82 % over all), the number of microspores that attained four or more nuclei, and the number of divisions per 100 alive microspores after 21 d of culture.

Additional key words: anther culture, 2,4-D, haploid, low temperature, × *Triticosecale.*

Introduction

The *in vitro* culture of anthers or isolated microspores is the method most often used to obtain haploid cereals. This technique has been used with wheat (Zhou and Konzak 1989, Hu and Kasha 1997, Tuvesson *et al*. 2000), rye (Deimling and Flehinghaus-Roux 1997), barley (Pickering *et al*. 1992, Cistué *et al*. 1994) and triticale (Charmet and Bernard 1984, González *et al*. 1997, González and Jouve 2000). The spontaneous or artificial duplication of chromosomes gives double haploid plants (DH), reducing the time required to obtain a homozygous line or cultivar. Further, such plants allow the expression of recessive alleles and are excellent material for basic studies, for example in the construction of genetic maps.

Despite the interest in androgenesis, recalcitrant species and/or genotypes exist that limit its use (Pelletier 1998). Many efforts have been made to increase the production of haploids via androgenesis, modifying factors such as the growth conditions of the anther donor plants, the culture medium, and environmental factors such as light, temperature and humidity. Genotype also influences in the androgenetic response (Henry and De Buyser 1985, Kasha *et al*. 1990, Jähne and Lörz 1995, González *et al*. 1997, Immonen and Anttila 2000, González and Jouve 2000, Puolimatka and Paul 2000, Smýkal 2000).

In cereals, the androgenetic process is divided into several stages controlled by independent genetic systems (Henry and De Buyser 1985, Knudsen *et al*. 1989, González *et al*. 1997). The first phase is the production of embryos or embryo-like structures (ELS) via the development of microspores. To achieve this, the normal development of microspores has to be blocked and turned towards sporophytic development. This can be achieved by different stresses treatment, *e.g*., subjecting the spikes or anthers to low temperature (Jähne and Lörz 1995), using mannitol to induce osmotic stress in the anthers (Roberts-Oëheschlager and Dunwell 1990, Cistué *et al*. 1994), or subjecting them to high temperatures and nutrient starvation (Touraev *et al*. 1996). Such treatments are often associated with the symmetric division of microspores (Zaki and Dickinson 1991, Hause *et al*. 1993). This is the opposite to that seen during gametophytic development, in which a vegetative and generative nucleus (macronucleus and micronucleus) are produced through asymmetric division. However, in some tree species such as *Aesculus hippocastanum*, no treatment of anthers to induce androgenesis has been applied (Ćalić *et al*. 2003).

Numerous studies have been published on the changes suffered by microspores when stimulated to undergo

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; DH - double haploid; ELS - embryo-like structures.

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sporophytic development (see Smýkal 2000, Shim and Kasha 2003). Nevertheless, no quantitative analysis of the development of microspores over time has been undertaken. The aim of the present work was to analyse the influence of genotype and culture medium on the development of microspores during the first 21 d of *in vitro* triticale anther culture. This work attempts to understand the reasons for the low embryos or ELS yield obtained from microspores.

Materials and methods

Two cultivars of hexaploid triticale (× *Triticosecale* Wittmack) Torote and Presto were used as anther donors, as well as their F_1 hybrid To×Pe. For the induction of *in vitro* androgenesis, spikes containing microspores at the mid-uninucleate stage were subjected to a temperature of 4 ºC for two weeks. After this period they were disinfected and cultured according to González *et al*. (1997). Two culture media were used: $N6_0$ and $N6_D$. Both were based on the medium N6 (Chu 1978), to which 100 mg dm^{-3} of inositol, 500 mg dm^{-3} of L-glutamine, 100 g dm^{-3} sucrose and 2.5 g dm^{-3} of *Phytagel* (*Sigma*, USA) were added. The two media differed in that $N6_D$ also contained 2 mg dm^{-3} 2-4 D.

At 0, 1, 2, 3, 5, 8, 10, 14 and 21 d of culture, anthers were collected from all three genotypes sown on the two culture media. These were then fixed in alcohol/acetic acid (3:1) and stored at 4 ºC until staining. To stain the microspores, anthers were immersed in a solution of acetic carmine (5 %) for 48 - 72 h. Once stained, the anthers were dissected on a microscope slide in a drop of acetic acid (45 %). A stereo-microscope and thin forceps were used to extract the microspores. To avoid their evaporation during observation under the light microscope, a coverslip was placed over them and the preparations sealed with *DePeX* (*Serva*, Germany). *Kodak QH* film was used for making photomicrographs.

Microspores with one or more stained nuclei were understood to be alive, and were examined in order to obtain a sufficient number of living microspores for each genotype and experimental condition (media and time of incubation). The number of nuclei in each of the microspores was recorded. Binucleate microspores were checked to see whether the two nuclei were the same (symmetrical division) or different (asymmetric division).

Results

After pre-treatment in the cold, all the microspores examined were in the mid- or late-uninuclear stage (Fig. 1*A*). From the beginning of culture it was observed that some microspores aborted (identified by their lack of stained nuclei) (Fig. 1*B*). From day 3 of culture, approximately one half of the microspores had aborted.

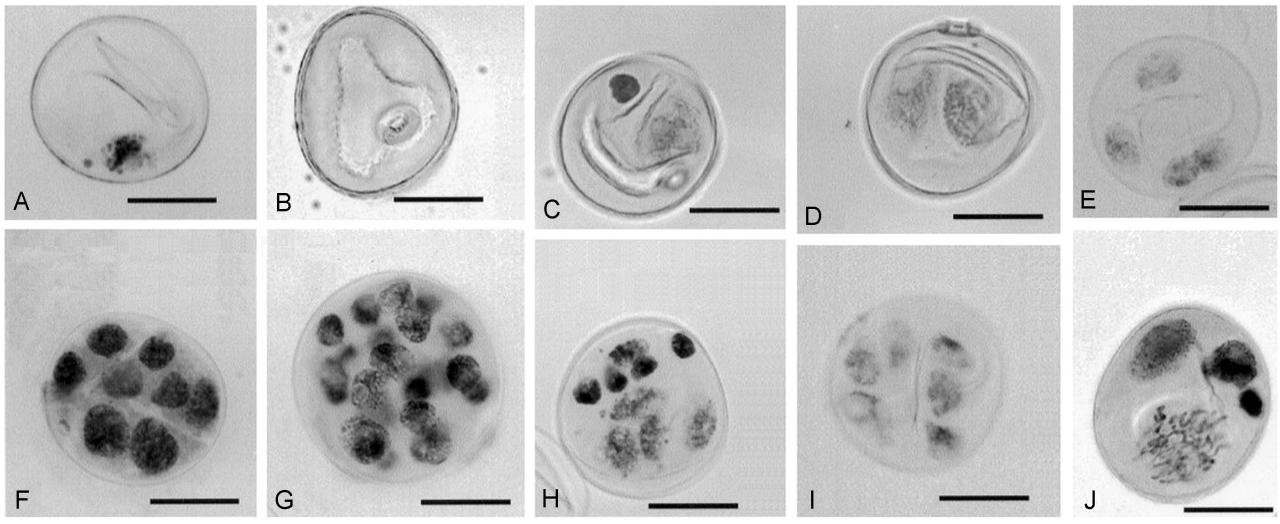

Fig. 1. *A* - microspore at the mid-uninucleate stage, *B* - aborted microspore lacking of stained nuclei, *C* - binucleate microspore showing asymmetrical division, *D* - binucleate microspore showing symmetrical division, *E* - microspore with 4 nuclei, *F* - microspore with 8 nuclei, *G* - multinuclear microspore showing more than 20 nuclei, *H*, *J* - microspores in which the nuclei have different state of condensation or division, respectively, *I* - microspore with a number of nuclei different to 2^n. *Bar*: 20 μm.

Table 1. Development of microspores in the *in vitro* culture of anthers on media $N6_0$ and $N6_D$. Tm - number of microspores studied; md - microspores died [%]; um - uninucleate microspores [%]; m2 - binucleate microspores [%]; m4 - microspores with four or more nuclei [%]; dm - number of divisions per 100 alive microspores (= number of nuclei-1).

Genotype	Culture	$N6_0$						$N6_D$					
	[d]	Tm	md	um	m2	m4	dm	Tm	md	um	m2	m4	dm
Torote	0	100	0	100	0	0	0	100	0	100	0	0	0
	1	126	20.63	100	0	0	0	114	12.28	100	0	0	0
	2	165	39.39	100	0	0	0	180	44.44	100	0	0	0
	3	170	41.17	99	1	0	1	191	47.64	91	9	0	9
	5	325	69.23	64	34	2	40	359	72.14	78	18	4	30
	8	777	74.26	64.5	29.5	0	41.5	695	85.61	55	42	3	51
	10	1654	87.91	78	20.5	1.5	25	1308	84.71	67	27.5	4	46
	14	2300	95.45	68	25	3	53	3599	92.91	71.4	23.9	4.7	45.0
	21	2963	95.37	90.5	5.1	4.4	69.3	5666	94.90	51.0	22.7	21.4	195.8
Presto	0	100	0	100	0	0	0	100	0	100	0	0	0
	1	110	10	99	0	0	1	116	12.28	100	0	0	0
	2	196	48.98	100	0	0	0	173	42.19	100	0	0	0
	3	205	51.22	93	7	0	7	384	47.92	65	35	0	35
	5	727	77.72	68.5	25.9	3.7	0	1806	70.87	58.5	30.8	9.9	54.9
	8	1432	83.03	68.7	28.0	0	37.0	783	74.46	55	37	4	64
	10	1298	82.66	64.7	24	2.7	36.4	415	75.90	65	22	11	67
	14	2510	96.01	70	19	11	97	1380	78.26	52	30.3	12.7	110.3
	21	3462	97.11	59	19	22	338	1158	82.73	46	26.5	25.5	259.5
To×Pe	0	100	0	100	0	0	0	100	0	100	0	0	0
	1	236	11.01	100	0	0	0	243	17.69	100	0	0	0
	2	136	26.47	100	0	0	0	159	37.11	100	0	0	0
	3	250	60.00	91	9	0	9	787	61.88	72	27.7	0.3	28.7
	5	923	78.33	48.5	51.5	0	51.5	842	64.37	67	31	2	41
	8	1502	86.55	51.5	40.1	5.9	70.8	1857	83.84	66.7	28	3.3	42.7
	10	3189	90.59	64.7	25	3.3	59.3	1864	88.52	66.3	21.5	10.3	98.1
	14	2025	90.12	73.5	15.5	8.5	95	1345	85.13	53.5	34.5	9	89.5
	21	2235	91.05	31.5	21	43	420	3194	87.44	47.6	15.7	35.7	410.2

This number increased to 82 - 97 % by day 21. Slightly more microspores aborted in anthers sown on $N6_0$ medium than on $N6_D$ medium (Table 1).

Table 2. Percentage of asymetric and symmetric divisions in the binucleate microspores that have appeared along the 21 d of *in vitro* culture of the anthers for each genotype and media.

Genotype	$N6_0$		$N6_D$	
	symmetric	asymmetric	symmetric	asymmetric
Torote	79.33	20.67	89.55	10.45
Presto	90.05	9.95	90.21	9.79
To×Pe	79.22	20.78	69.25	30.75
Total	82.55	17.45	83.30	16.70

To determine the percentage of asymmetric (Fig. 1*C*) and symmetrical divisions (Fig. 1*D*), the binucleate microspores that appeared along the 21 d of culture in each medium and for each genotype were counted (Table 2). The percentage of symmetrical divisions was very similar in both media. However, differences were seen between genotypes, with Presto showing 10 % more symmetrical divisions than the other genotypes, both on $N6_0$ and $N6_D$ medium. Practically in all cases, microspores with two nuclei appear from day 3 of culture, with 4 nuclei (Fig. 1*E*) begin to appear from day 5 and with 8 or more nuclei (Fig. 1*F*,*G*) do not appear before 8 d (Table 1).

The percentage of uninucleate microspores diminished as culture time progressed, with an important decrease on day 5. This agrees with the increase in the number of binucleate microspores. Between days 8 and 10 there was a recovery in the percentage of uninucleate microspores and a decrease in binucleate and plurinucleate microspores, respect to the alive microspores. The $N6_0$ medium produced a greater number of uninucleate microspores and the $N6_D$ medium produced more microspores with 4 or more nuclei. Differences were also observed between the three genotypes, especially at 21 d of culture.

In almost all plurinucleate microspores, the nuclei looked similar, though some showed different states of condensation or division (Fig. 1*H*,*J*). Great variation was observed in the number of nuclei in microspores coming

from the same anther, due to asynchrony in their development. Some microspores, neither was there synchrony in the divisions of the nuclei, demonstrated by the appearance of microspores with non-2^n nuclei (Fig. 1*I*). The highest number of divisions per 100 alive microspores was observed on medium $N6_D$ than on $N6_0$. With respect to genotype, To×Pe showed the greatest number of divisions.

Discussion

One of the problems in *in vitro* androgenesis in triticale, is the low number of embryos obtained. Thus, the best genotypes of triticale analysed produce only 1 or 2 ELS per anther (González *et al*. 1997) despite the 4000 - 4500 microspores per anther. Studies have been performed to determine the destiny of microspores in wheat, in order to help identify embryogenic microspores capable of producing haploid embryos (Indrianto *et al*. 2001). However, there has been little work on the quantitative aspects of embryo development, *i.e.*, analyses of microspore division patterns during culture. Such an analysis – the aim of the present paper – would help to reveal the influence of genotype and culture medium on the development and final destiny of microspores.

The first step in androgenesis is to obtain embryos or ELS from microspores. For this, the microspore has to be turned from gametophytic development towards an embryogenic pathway, which can be achieved through stress (Pechan *et al*. 1991). Zheng and Ouyang (1980) reported that cold pre-treatment induces symmetrical division in wheat microspores. However, in wheat anther culture, Hassawi *et al*. (1990) report that the first division of microspores is asymmetric, unlike in the culture of isolated microspores in which the first division (after 3 d of culture) is symmetrical (Bonet and Olmedilla 2000). In triticale, the stress signal used has been cold pre-treatment of spikes that contain microspores at the mid-uninucleate stage (Charmet and Bernard 1984, González *et al*. 1997, Tuvesson *et al*. 2000), but until now, no studies had been performed to determine the type of division induced in the microspores.

The present work shows that most microspores underwent the first symmetrical division in both culture media ($N6_0$ = 82.55 % and $N6_D$ = 83.30 %) for all three genotypes. Nevertheless, the separate analysis of each genotype shows differences in their behaviour in these media. In terms of the percentage of microspores that undergo symmetrical division, our study shows that 2.4-D in the culture medium influences the three genotypes to different extents. But, if the induction of symmetrical division was the only factor responsible for the final production of ELS, Presto should have been the genotype that produced the most and To×Pe the least. González *et al*. (1997), who studied the production of ELS in these three genotypes, observed that To×Pe produces the greatest number of ELS followed by Presto and Torote. In view of these results, other factors must also determine ELS formation.

The number of microspores that abort during the culture might explain much about the ELS production rate. To investigate this, the percentage of microspores that did not stain with acetic carmine (aborted microspores) was examined over the culture period. An increase in the percentage of aborted microspores was seen as culture time progressed (Table 1). Similar results have been observed in *Solanum* (Říhová and Tupý 1999) and barley (Wojnarowiez *et al*. 2002). Generally, more microspores survived on $N6_D$ medium than on $N6_0$. Differences were also seen between the genotypes, but mainly on $N6_0$ medium.

In the present study, the analysis of microspore divisions during triticale anther incubation indicates that the first divisions occur from the third day of culture, although the majority of microspores continue in a uninucleate state. After 5-d culture, microspores with four nuclei begin to appear and from the tenth day, microspores arise with more than 16 nuclei. An increase in the percentage of uninucleate microspores respect to the alive microspores was observed between day 5 and 8, as well as a decrease in binucleate and plurinucleate microspores. This result might be explained by the death of the microspores in divisions, a phenomenon observed in wheat by Szakács and Barnabás (1995). This might help explain the low production of ELS in wheat and triticale.

An important point is the percentage of microspores that contain 4 or more nuclei: these microspores have been clearly separated from the gametophytic pathway. This percentage is greater in anthers sown on medium $N6_D$ than on $N6_0$ in all three genotypes, although intergenotypic differences were more evident when the microspores came from anthers that remained in culture for 21 d (Table 1). The hybrid To×Pe showed the greatest percentage of microspores with 4 or more nuclei, followed by their parents Presto and Torote. Similar results were observed when the number of divisions per 100 alive microspores was analysed. This heterotic effect has been observed in the androgenetic response for these genotypes (González *et al*. 1997).

One of the constants in the *in vitro* culture of anthers or microspores is the lack of synchrony in development. In triticale, asynchrony in microspore development is quite evident. For instance, in anthers maintained in culture for 21 d, a great number of aborted microspores were seen, together with microspores in the uninucleate, binucleate, plurinucleate and ELS which contained 100 or

more nuclei or cells. This lack of synchrony is equally evident within the same microspore, since non 2^n nuclei appear (Fig. 1*I*), as well as microspores with nuclei in different states of division (Fig. 1*J*). The lack of synchronization in the development of the microspores could be a disadvantage in studies that require markers of different stages of the sporophytic pathway.

In conclusion, this study indicates that the following are all dependent on genotype and culture medium: *a*) the microspore survival rate in the *in vitro* androgenesis of triticale, *b*) the percentage of symmetrical divisions, *c*) the number of divisions per 100 alive microspore, and *d*) the number of microspores with 4 or more nuclei. Finally, a high percentage of microspores in culture aborted, which explain the low success rate obtained in *in vitro* androgenesis of triticale (and probably of other cereals). The presence of 2.4-D in the culture medium favoured microspore division and survival. The success rate of *in vitro* androgenesis could be improved by reducing the rate of abortions of microspores, *i.e.*, by changing the culture conditions, incubation temperature (Smýkal and Pechan 2000), or some of the components of the culture media Wojnarowiez *et al*. 2002).

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