Comparison between two methods of haploid production in hexaploid triticale (*X. Triticosecale Wittmack*)

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Abstract

In triticale breeding programs based on the doubled haploid technique, the rate of obtained haploid plants is important. Seeds of the F1 generation are costly and available only in small quantities. In this study, two techniques have producing haploid plantations in triticale haggard been compared; thesis were: in vitro androgenesis through anther culture and, the intergeneric crossing with maize.

The result indicated that the in vitro androgenesis method was superior despite a high rate of obtained albinos. This method had a higher performance in producing haploid plants, with an average of 3.5 haploid plants produced per cultured spike. On the other hand, even if producing haploid plants through crossing with Maize was less performing than anther culture overall, all plants regenerated were green. A possible explanation of this finding lies probably in that the lower fertility rate is linked to incompatibility mechanisms, thus resulting in a low (0.38) average number of obtained plants per fertilised spike.

The number of available F1 seeds is important for the genetic variability and hence for the potential recombination of traits. It is recommended that breeders use the androgenesis method.

Key words : Triticale; *in vitro* androgenesis; intergeneric cross; doubled haploid.

ملـخص

تم خلال هذا البحث مقارنة تقنيتين لإنتاج نبات أحادي الكروموزومات عند التريتيكال وهما: زراعة المآبر والتزاوج مع الذرة.

أظهرت هذه الدراسة انه بالرغم من وجود نبات أبيض غير طبيعي فإن تقنية زراعة المآبر هي المناسبة وذلك بمعدل إنتاج 3,5 نبتة أحادية الكروموزومات مع كل سنبلة يتم زرعها في الوسط الكيمائي.

ينتج التزاوج مع الذرة نباتا أحاديي الكروموزومات بنسبة أقل مقارنة مع زراعة المآبرمع خلوه من النبات الأبيض الغير الطبيعي.

بالنسبة لبرنامج التحسين الو راثي للتريتيكال والذي يعتمد على تقنية مستنسخ أحادي الكروموزومات فان عدد النبات المنتج جد مهم وذلك نظرا للعدد المحدود من الهجين الأول. لذلك ننصح باستعمال تقنية زراعة المآبرمع العمل على تحسين الوسط الكيمائي للحد من انتاج النبات الأبيض الغير الطبيعي.

الكلمات المفتاحية : التريتيكال، نبات أحاديي الكروموزومات، التزاوج مع الذرة.

Résumé

La comparaison de deux techniques de production de plantes haploïdes, l'androgénèse in vitro et le croisement intergénérique avec le maïs, a été conduite sur le triticale hexaploïde.

Les résultats ont montré qu'en dépit du taux d'albinisme élevé, l'androgénèse in vitro est plus performante comme méthode de production d'haploïdes, avec une moyenne de 3.5 plantes haploïdes par épi mis en culture.

La production de plantes haploïdes par croisement avec le maïs reste inférieure à celle par culture d'anthère, bien que la totalité des plantes régénérées soient chlorophyllienne.

Le faible taux de fécondation dû probablement à des mécanismes d'incompatibilité fait que le nombre d'embryons sauvés et par conséquent celui de plantes haploïdes ne dépasse pas 0.38 plantes par épis castré.

Dans un programme d'amélioration génétique du triticale utilisant l'haplodiloïdisation comme méthode de création variétale, le nombre de plantes haploïdes produites est très important car la quantité de semences F1 est faible et coûteuse. Il est donc recommandé en cas d'application de l'haplométhode d'utiliser la culture d'anthère avec toutefois le transfert rapide des embryons sur des milieux de régénération améliorés.

Mots clés : Triticale ; androgenèse *in vitro* ; croisement intergénérique ; haplodiploïdisation.

Introduction

Triticale (*X. Triticosecale Wittmack*), is a man made species produced through crossing between durum wheat and rye (hexaploid triticale) or between bread wheat and rye (octaploid triticale). The first cross (wheat x rye), obtained were sterile (Wilson, 1976) or partially fertile. With the discovery of colchicine, it became possible to routinely produce fertile hexaploid triticale by doubling the number of chromosomes. This technique permitted crossing between different genotypes of wheat and rye and therefore, the exploitation of a new genetic variability that allowed this new species to be an alternative crop beside wheat and barley in some stressed environments. Indeed, triticale tolerates acid and poor soils, and resists better to many different biotic and abiotic stress than the other small grain cereals. In addition, triticale responds well favourably to fertiliser application and the use of low doses of nitrogen and phosphorus outputs an optimum production (Mergoum et al., 1992).

Since with its self pollinated crop status triticale tolerates consanguinity, the haplodiploidisation or double haploid method is alternative to the classic techniques of self pollinated plants improvement or selection (Pedigree, SSD or Bulk) and can be used in variety creation. This method presents the advantage to fix lines in a short time.

In the same way and in the case of a limited introduction from the rye genome (addition, substitution or translocation lines), the application of the double haploid method permits fixation to homozygoucity state in only one generation.

The first studies on double haploid production of triticale used the in vitro androgenesis as method of haploid production. This technique permits good embryogenesis but plant development is characterised by the production of a very large number of albino plants (Ono et later, 1976; Bernard, 1977). This phenomenon could be explained by the presence of the rye genome (R), whose *in vitro* androgenesis is marked by the high percentage of albino plants (Wenzel et al., 1977; Marciniak et al., 1998). In triticale microspore culture, albinism remained a serious problem, which was reflected by occurrence of more than 50% of the regenerates being albinos (Monostori et al., 1998).

With other methods of haploid production in triticale, albinos plants do not generally occur. Crossing with *Hordeum bulbosum* is subject to incompatibility problems (Snape et al 1979.) but fertilizing with maize pollen can be considered as an alternative method for haploid production. Maize is insensitive to action of *Kr1* and *Kr2* alleles for incompatibility with rye genome.

The objective of this study is to compare two techniques of haploid production in triticale, namely *in vitro* androgenesis and intergeneric crossing with maize. This comparison will permit to identify the most efficient and most economic technique for triticale genetic improvement in the breeding programs of INRA, Morocco.

Material and methods

The plant material is constituted of twenty F1 hybrid of hexaploid triticale (*X. Triticosecale Wittmack*) provided by the International Center for Wheat and Maize Improvement (CIMMYT int) Mexico City D. F. Mexico (Table 1) and a Moroccan maize variety (Doukkalia). The triticale F1 hybrid plants were grown in the greenhouse. Planting was made at different dates in order to coincide their flowering period with that of maize.

Table 1. Cross name and pedigree of 20 hybrids used in the comparison.

Hybrid	Cross name and pedigree
1	TCB153WG/CMH77.1135/CMH77A1165//2*YOUGUI/3/IBEX/4/JLO97/CIVET
2	TCB153WG/3/DAGRO/IBEX//CIVET-2
3	TCB153WG//ERIZO610/BULL-1-1
4	TCB153WG//ERIZO-15/FAHAD-3
5	TCB153WG/FAHAD-5
6	TCB153WG/FAHAD-6
7	TCB153WG/GNU/ASAD//ARDI/3/MANATI-1
8	TCB153W/GLAMB-2
9	TCB153WG/MANATI-1
10	TCB153WG/5/PIKA-1/3/EDA-7//M2A/ZA75/4/GATO
11	TCB153WG/3/PRESTO//2*TESMO1-/MUSX-603
12	TCB153WGRHINO-3/BULL-1-1
13	TCB153WG/3/RONDO/BANT-5//ANOAS-2
14	TCB153WG/SONNI-3
15	TCB153WG/POLLMER-2.1.1
16	TCB153WG/POLLMER-2.2.1
17	TCB153WG/3/TESMO-1/MUSX-603//FAHAD-4
18	TCB153WG//ANOAS-3/TATU-4
19	TCB153WG//BULL-10/MANATI-1
20	TCB153WG/CAAL

For *in vitro* androgenesis, spikes were cut when microspores were at the mid-uninucleate and vacuolate stage of development and were pretreated for 7 days at 3°C in the dark (Picard and De Buyser, 1975). The spikes were sterilised in a 4% calcium hypochlorite solution for 3 minutes and rinsed with sterile water. The anthers were cultured under aseptic conditions on C17 anther culture medium (Wang and Chen, 1986). After incubation at 24°C in the dark, the embryos that were developed out of the cultured anthers were transferred on R9 regeneration medium (Miller, 1963). The albino plants were counted, and eliminated.

For intergeneric crossing with maize, the method adopted in this study was described in bread wheat (Laurie and Bennett, 1986). Spikes were emasculated two to four days before anthesis and covered with glassine bags. The spikes were pollinated with freshly collected

maize pollen. At the same time, a solution with 100 mg l^{-1} of 2,4-D (2,4-dichlorophenoxy-acetic acid) is injected in the upper cavity of the last inter node. One day after pollination a second injection of the 2, 4-D is applied as well as the spraying of 75 mg l^{-1} of gibberellic acid (GA) on the spike.

Two weeks after pollination, spikes were collected. Seeds were extracted from spikes and surface sterilised in a (4% calcium hypochlorite solution for 1 minute, 70% ethanol for 1 minute) and then rinsed with sterile distilled water. Embryos obtained were aseptically excised and transferred on Murashige and Skoog medium (Murashige and Skoog, 1962) in plastic petri dishes and incubated at 24°C in the dark. After germination, plantlets were transferred to pots and placed in the greenhouse.

The green plants with developed roots obtained from both methods were transplanted to soil and grown in greenhouse. The chromosome numbers of the cultured plants were counted in root tips using the standard feulgen technique. Haploid plants were treated with 0.2% colchicine, 1% DMSO for 4 hours, washed and replanted in order to obtain fertile double haploid plants.

Statistical analysis was made in anther culture for the following traits: percentage of embryos expressed per 100 anthers cultured, green plants and albinos plants expressed per 100 anthers cultured.

For the cross with maize, statistical analysis was made for the following traits: percentage of caryopses, embryos and green plants expressed per 100 pollinated florets.

The analysis of variance was made with the genotype as the source of variation in a randomized design. The separation of means was achieved by Duncan test with LSD at 5% level of probability.

Results and discussion

The analysis of the variance showed a very highly significant effect of the genotype on the percentage of embryos, green plants and albino plants in anther culture (Table 2).

Table 2. Analysis of variance: effect of genotype on the percentage of embryos, green plants and number of albino plants.

Source of variation	df	% Embryos	% green plants	% albinos plants
Genotype	19	4424,12 ***	382,54 ***	815,5 ***
Error	20			

** * *, significant at the 1 %° level.*

Differences between genotypes were also important: the percentage of embryos varied from 0% to 215,5% (genotype 8). Green plant percentages varied from 0% to 37,5% (genotype 3). The overall mean of embryos percentage is 66,2%, the overall mean green plants percentage is 8,3% and the overall mean albino plants percentage is 18% (Table 3).

Table 3. Results of in vitro androgenesis obtained by culturing anthers from 20 triticale F1 hybrids.

Hybrid	Spikes used	Anthers cultured	Embryos obtained		Green Plants		Albinos Plants	
			Number	%	Number	%	Number	%
1	4	168	24	14,3 j	4	2,4 g	16	9,5 i
2	2	84	20	23,8 i	2	2,4 g	14	16,7 h
3	4	168	97	57,7 g	22	13,1 e	44	26,2 e
4	4	168	63	37,5 h	3	1,8 g	14	8,3 ij
5	2	84	0	0 l	0	0 h	0	0 l
6	3	126	97	77 f	1	0,8 gh	9	7,1 j
7	2	84	91	108,3 d	2	2,4 g	20	23,8 f
8	2	84	181	215,5 a	16	19 d	25	29,8 d
9	2	84	110	131 c	26	31 a	40	47,6 a
10	2	84	8	9,5 k	0	0 h	0	0 l
11	2	84	93	110,7 d	1	1,2 gh	0	0 l
12	4	168	162	96,4 e	24	14,3 e	59	35,1 c
13	3	126	10	7,9 k	3	2,4 g	5	4 k
14	2	84	8	9,5 k	0	0 h	0	0 l
15	2	84	148	176,2 b	5	6 f	21	25 ef
16	2	84	33	39,3 h	0	0 h	0	0 l
17	2	84	64	76,2 f	16	19 d	41	48,8 a
18	2	84	66	78,6 f	21	25 c	34	40,5 b
19	3	126	121	96 e	36	28,6 b	27	21,4 g
20	3	126	50	39,7 h	0	0 h	26	20,6 g
Total	52	2184	1446		182		395	

LSD (percentage of embryos) = 2,6, LSD (green plant percentage) = 0,54, LSD (percentage of plants albino) = 0,57.

Percentages followed by the same letter are not significantly different at 0.05 probability by Duncan LSD test

The embryos production is satisfactory, but the number of green plants is low. Only 182 green plants have been regenerated for more than 1446 embryos. In the same way, a high percentage of albino plants is to be noted. The whole 182 green haploid plants were produced from 52 cultured spikes, which represents an average of 3,5 green haploid plants per spike. The analysis of variance of seed set, number of embryos and green plants showed a very highly significant effect of the genotype (Table 4).

Table 4. Analysis of variance: effect of triticale genotype on seed set, embryos and number of green haploid plants.

Source of variation	df	% Seed set	% Embryos	% Haploid plants
Genotypes	19	37,7 ***	46,19 ***	12,72 ***
Error	20			

***, *significant at the 1 %° level.*

The intergeneric cross with maize were marked by a low caryopses set percentage (6,3%) (Table 5). Variations between genotypes were between 0% and 30% (genotype 20), and the absence of albinos plants is noted. Indeed, from 37 rescued embryos, 21 green plants were obtained. The number of embryos and green plants varies according to genotypes of F1 hybrids. All the 21 green haploid plants are gotten from 54 emasculated spikes, which represents an average 0,38 green haploid plants per spike. The repetition number must be increased in order to obtain more caryopsis and embryos.

If the intergeneric crossing with maize is considered as an alternative method for haploid plant production in recalcitrant species to *in vitro* androgenesis, the case of triticale, as shown in this study, is to treat otherwise. Triticale, due to its hybrid structure (interspecific origin), has two different responses for the two haploid plants production methods used.

Table 5. Numbers and percentages of seed set, embryos and haploid plants obtained from crossing 20 triticale F1 hybrids with maize.

Hybrid F1	Spikes emasculated	Florets pollinated	Caryopses developed		Embryos obtained		Green plants	
			Number	%	Number	%	Number	%
1	2	40	0	0 g	0	0 d	0	0 d
2	4	80	7	8,8 def	3	3,8 e	1	1,25 cd
3	2	40	7	17,5 b	4	10 b	3	7,5 b
4	2	40	5	12,5 cd	1	2,5 cd	0	0 d
5	2	40	0	0 g	0	0 d	0	0 d
6	4	40	0	0 g	0	0 d	0	0 d
7	2	40	0	0 g	0	0 d	0	0 d
8	2	40	5	12,5 cd	4	10 b	3	7,5 b
9	4	80	11	13,8 bc	2	2,5 cd	1	1,25 cd
10	2	40	0	0 g	0	0 d	0	0 d
11	2	40	0	0 g	0	0 d	0	0 d
12	2	40	0	0 g	0	0 d	0	0 d
13	4	80	4	5 f	2	2,5 cd	1	1,25 cd
14	2	40	5	12,5 cd	2	5 c	1	2,5 cd
15	2	40	3	7,5 ef	2	5 c	0	0 d
16	4	80	9	11,3 cde	8	10 b	4	5 bc
17	2	40	0	0 g	0	0 d	0	0 d
18	2	40	0	0 g	0	0 d	0	0 d
19	2	40	0	0 g	0	0 d	0	0 d
20	2	40	12	30 a	9	22,5 a	7	17,5a
Total	54	1080	68		37		21	

LSD (percentage of seeds) = 13,96., LSD (percentage of embryos) = 2,47, LSD (percentage of plants) = 3,6.

Percentages followed by the same letter are not significantly different at 0.05 probability by Duncan LSD test.

In *in vitro* androgenesis, the hybrid vigour especially appears in embryogenesis haploid (Figure1). Some genotype had 215.5% in percentage of obtained embryos (genotype 8).

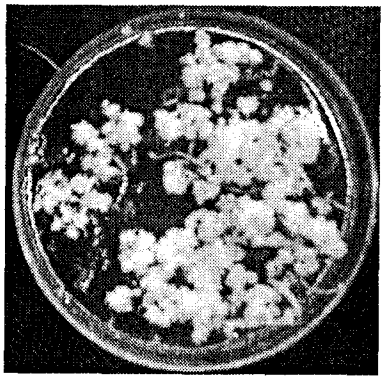

Figure1. Embryoid formation obtained from triticale anthers cultured on C17 medium.

The crossing with the maize generates a low rate of seed set probably due to mechanisms of incompatibility that appear in species descending from interspecific hybridization. This may explain the low rate of seed set observed in this study: 6,3 seeds set for 100 flowers pollinated. These results could be confirmed or improved by an ulterior study with enough repetition.

The anthers culture technique is simpler than crossing with maize which requires, in addition, the adjustment of flowerings periods of the two species and their genotypes. The period of time between the planting and germination of intended plants to the haploid production and the production of doubled seeds is appreciably even for the two methods: this period is between 9 and 11 months. Considering this results, crossing with maize does not offer an attractive alternative for the *in vitro* androgenesis in triticale.

The bottleneck of the plants haploid production through anther culture remains the green plants development. A lot of progress is achieved by the improvement of environmental conditions of culture, with new sources of carbon and nitrogen (Marciniak and al., 1998) that permitted the production of embryos in large numbers. Much effort must be also made to improve surrounding conditions of regeneration stage in order to obtain more green plants. Embryos obtained from anther culture are transformed quickly to unorganized callus and necrosed, their fast transfer on regeneration medium could increase the number of green plants.

In bread wheat, the use of single regenerated medium which plantlets are regenerated directly from anthers and also improved the frequencies of green plants regeneration (Lashermes, 1992). We observed the same phenomenon in triticale, green haploid plants could be effectively produced via anther culture with indol acetic acid (AIA), instead of 2,4-D (Figure 2). However, culture - regenerated medium should be improved.

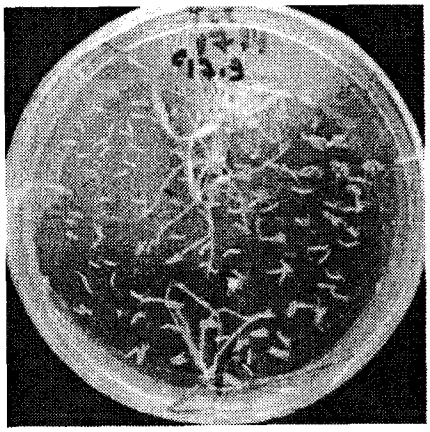

Figure2. Direct regeneration from triticale anthers.

Conclusion

In spite of the problem of albinism and the low rate of regeneration, the *in vitro* androgenesis remains the most advantageous method for producing haploid plants in triticale. With a satisfactory embryogenesis, regeneration must be improved to increase the number of viable haploid plants necessary to recover a useful genetic variability prior to plant selection and evaluation process.

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