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Production of wheat doubled haploids by *in vitro* anther culture

The *in vitro* culture response of anthers, originating from high yielding and resistance to lodging F₁ soft wheat hybrid plants selected under drought conditions, was investigated. The spikes were collected when the microspores were at the mid-uninucleate stage and after 3 days at 4^o C for cold pre-treatment, the anthers were cultured in N6 and Gamburg-Eveleg B5 (B5) solid medium. Progeny from 17 different F₁ families were used and 9 of them responded to *in vitro* anther culture and 7 of them produced fertile green plants. Doubled haploid green plants were fertile and produced seeds. Induction of haploid development depends on genotype, stage of male gametophyte development, composition of nutrient media and conditions of cultivation. The effect of the composition of media and different genotypes on wheat anther culture was investigated. From the genotypes produced regenerants, the high yielding genotypes from local breeding collection of wheat produced more albino plants (35.2%) compared to high yielding genotypes from CYMMIT collection (24.92%) ones. Independent of culture medium composition a ratio of doubled haploid/haploid was 4.57:1 and a ratio of haploid/aneuploid was 18.5:1, in particular 81.28 % of green plants were doubled haploid, 17.76% were haploid and the rest (0.96%) were aneuploid

Key words: Anther culture, wheat, morphogenesis, doubled haploids, drought resistance

Introduction

The production of homozygous plants is a difficult and long procedure. Conventional methods of plant breeding need many cycles of selection to isolate promising genotype and genetically constant lines. When the conventional method is used, this procedure takes approximately twelve to fifteen years. Anther culture technique accelerates the breeding cycle by shortening the time required to attain homozygosity [1]. Double haploid techniques provide plant breeders with pure lines in a single generation. The use of elite lines as crossing parents combines the opportunity to select more efficient agronomic traits in homozygous plants. Therefore, double haploid breeding strategies have competitive advantages compared with conventional methods [2]. When the F₁ is used, this technique has the advantage that genetic uniformity is achieved only in a short time after the initial hybridization. Anther culture allows a rapid production of appropriate genotypes for breeding purposes in an effort to identify promising pure lines [3]. The production of double haploids through androgenesis represents a modern tool for the improvement of cultivated species. Developmental stage of harvested spikes, cul-

ture medium, duration of cold pretreatment and genotype may affect haploid induction in anther culture whereas among them genotype play a major role in embryo and green plant production in cereals [4]. The problem affecting the efficiency of androgenesis is the production of albino plantlets in various proportions according to the cultivars and the low numbers of plants that are regenerated. Therefore, further improvement in anther culture efficiency in wheat is needed. The target of this study was to determine optimal composition of culture medium for haploid production in different wheat genotypes.

This paper presents evidence of a beneficial effect of N6 culture media for wheat anther, resulting in significant increases in green plant regeneration and impact of genotypes on induction embryogenesis and regeneration in wheat anther culture.

Materials and methods

Donor Plant materials

17 groups of spring wheat (*Triticum aestivum* L.) plants were selected and finally we had 8 different families. Progeny of 8 different F₁ soft wheat plants (families) were used for *in vitro* anther cul-

ture. The hybrids of soft wheat were produced from the following crosses: *Lutescens*293H436 x *Gostianum* 88 (BG 1-4/98), (*Gostianum*88 x *Lutescens* 410H48) x *Lutescens* 410H48 (BG 1-3/98), wheat hybrids from CYMMIT SN64//SKE12* ANE/3/SX/4/BEZ/5/JUN ARAPAHOE (BG 367), DAGDAS/6/ SN64// SKE12* ANE/3/SX/4/BEZ / 5/JUN (BG 357), hybrids obtained with izogenic line Lr24 – *Kazakhstanskaya* 17 x Lr 24 (BG-L3), K49397 x Lr 24 (BG-L9), (*Spartanka* x *Erytrospermum* 14) x *Erytrospermum* 14 (BG 7-2/97, Line 86 x *Celinaya* 24 (BG 6-7/97), (*Mironovskya* 808 x *Skala*) x *Skala* (BG 6-8/97). The donor plants were resistant to drought, leaf yellow rust and lodging.

Growth conditions of donor plants

Seeds of each family were sown in rows 3 m long within the last week of April in an experimental field at the Institute of Plant Biology and Biotechnology in south-east Kazakhstan. The experiment was established in a chestnut soil and organic matter content 1.2% (10 to 30 cm depth). All the common cultivation practices were used. Spikes of the plants were harvested for anther culture when the sheath of the flag leaf had emerged about 0.5 cm (it is equivalent to one-nuclear stage of microspores), and remained at 4°C for 3 days for cold pretreatment.

Anther culture conditions

The anthers whose the microspores were at the mid uninucleate stage, were excised aseptically from the spikes. The spikes were previously sterilized with 70% ethanol. The anthers were excised and inoculated into petri-dishes (60x15 mm) containing 3 ml N6 and Gamborg-Eveleg B5 (B5) medium solidified by agar, supplemented with 1 mg/l 2,4-D. The culture medium including 100 mg/l myo-inositol, 9% (w/v) sucrose.

Each petri-dish contained approximately 90 anthers. The anthers were incubated in the dark at 25°C during 2-4 weeks (the anthers were cultured for a further 2 weeks before first observations were made). The embryos produced were transferred to a regeneration medium and incubated in the light (at 40-80 $\mu\text{mol}/\text{m}^2/\text{sec}$) under a 16h photoperiod at 25±2°C for 4 weeks. The regeneration medium used was N₆ solid medium, supplemented with 2% sucrose, 1.5 mg/l IAA and 1 mg/l kinetin. The root meristems of plantlets were used to determine the ploidy level (to determine the number of chromosomes of the obtained green plants). The roots were pretreated with 8-Hydroxyquinoline for three hours and fixed in 3:1 acetic alcohol. After a few days the

chromosomes were stained by the Feulgen technique and slides were prepared in acetocarmine.

Green plantlets were transplanted in small pots covered by plastic bags to maintain the high humidity (60-70% humidity). After 2-3 weeks the plants were transferred to larger pots and kept at 25⁰/16⁰ C day/night temperature regime with 16h photoperiod to reach the maturity and produce seeds. Seeds from the families produced green plants were treated properly to stain and count the chromosome number.

Data were recorded on the basis of morphogenesis and embryogenesis (morphogenetic structures or embryo per 100 anther), regeneration (albino and green regenerated plants per 100 anthers) and diploidization (total double haploids). The statistical analysis was computed following the working schedule of Lakin (1990) [5]. Differences were considered significant at P=0.05.

Results and Discussion

From 17 families used in anther culture only 9 of them (52.94%) responded and produced morphogenic callus and embryos (Table 1) (Fig. 1A, 1B). From those 9 families, 4 originated from high yielding genotypes from CYMMIT (BG 357, BG 367) and wheat hybrids obtained by breeders from Institute of Plant Biology and Biotechnology. Five wheat hybrids originated from middle yielding genotypes. Furthermore, significant differences were found among genotypes originated from high yielding (3,53-10,18% embryos per 100 anthers) and middle yielding plants 1,11-6,48% embryos per 100 anther). Concerning the regeneration, 7 of the responded genotypes produced plants (Table 2) (Fig. 1C, 1D). On the other hand, 3 genotypes produced both green and albino plant-regenerants. The ratio between green and albino plants were 5,99:1 for hybrid BG 1-3/98, 1,25:1 for BG 1-4/98 on the N6 culture medium and 1:1 for genotype BG 367 on the B5 culture medium. The anther cultivation on N6 medium induced more androgenic structures (morphogenic callus+embryoids) compared to B5 culture medium for all responded wheat genotypes.

Furthermore, from the genotypes produced regenerants, the high yielding genotypes from local breeding collection of wheat produced more albino plants (35.2%) compared to high yielding genotypes from CYMMIT collection (24.92%) ones. All of green plants grown on the N6 culture medium were fertile and produced seed. The cyto-genetical

analysis showed that the 81.28 % of green plants were doubled haploid, 17.76% were haploid and the rest (0.96%) were aneuploid.

Most of the families produced embryoids, morphogenetic callus and regenerants originated from high yielding genotypes. The differences between high yielding and middle yielding genotypes in embryoid production and green plant grown on the

same medium may be due to genotypes because both androgenic response and regeneration capacity were greatly genotype dependent. A major problem with anther culture in cereals is the low numbers of plants that are regenerated. Often this problem is compounded by a high frequency of albinos. Mean differences among different genotypes presented $P \leq 0.05$.

Table 1 – Influence of medium composition on the morphogenesis in anther culture of wheat

Hybrid	Culture Medium	Number of anther	Morphogenic callus, %	Embryoids, %	Total number of androgenic structure, %
BG 1-3/98	N6	522	1,34±0,05	3,06±0,07	4,40
	B5	594	0,67±0,03	3,53±0,08	4,20
BG 1-4/98	N6	306	1,30±0,06	2,94±0,09	4,24
	B5	252	0,39±0,03	1,58±0,07	1,97
BG 357	N6	108	0	10,18±0,29	10,18
	B5	72	4,16±0,23	4,17±0,24	8,33
BG 367	N6	126	1,59±0,01	1,59±0,11	3,97
	B5	126	0,79±0,07	2,38±0,13	3,17
BG-L 9	N6	108	1,85±0,09	5,55±0,22	10,17
	B5	126	0,79±0,07	0,79±0,07	1,58
BG-L 3	N6	108	0	6,48±0,24	6,48
	B5	126	0	3,97±0,17	3,97
BG 7-2/97	N6	144	7,64±0,02	4,86±0,02	13,88
	B5	180	1,11±0,01	1,11±0,01	2,22
BG 6-7/97	N6	126	1,59±0,01	2,38±0,01	7,14
	B5	90	5,55±0,02	3,33±0,02	8,88
BG 6-8/97	N6	216	4,63±0,01	4,16±0,01	11,57
	B5	234	0,85±0,06	0,85±0,06	2,14

Table 2 – Number of embryoids, androgenic structures, albino and green plants production after *in vitro* anther culture of spring wheat

Genotype	Culture Medium	Number of anther	Number of androgenic structure, %	Number of regenerants, %		Total number of plantlets, %
				green	albinos	
BG 1-3/98	N6	522	4,40±0,12	26,09±0,93	4,35±0,43	30,43±0,98
	B5	594	4,20±0,11	20,00±0,82	0	20,00±0,82
BG 1-4/98	N6	306	4,24±0,15	38,8± 1,40	30,95±1,4	69,75±2,80
	B5	252	1,97±0,10	60,1±2,40	0	60,1±2,40
BG 357	N6	108	10,18±0,29	36,36±1,52	0	36,36±1,52
	B5	72	8,33±0,47	33,33±2,11	0	33,33±2,11
BG 367	N6	126	3,97±0,19	50,0±2,80	0	50,0±2,80
	B5	126	3,17±0,20	24,92±4,30	24,92±4,3	49,84±8,60
BG -L 9	N6	108	10,17±0,46	37,3±1,70	0	37,3±1,70
	B5	126	1,58±0,14	100,0±0	0	100,0±0
BG -L 3	N6	108	6,48±0,24	42,70±2,00	0	42,70±2,00
	B5	126	3,97±0,17	20,00±2,00	0	20,00±2,00
BG 7-2/97	N6	144	13,88±0,03	15,0±0,08	0	15,0±0,08
	B5	180	2,22±0,01	50,0±0,28	0	50,0±0,28

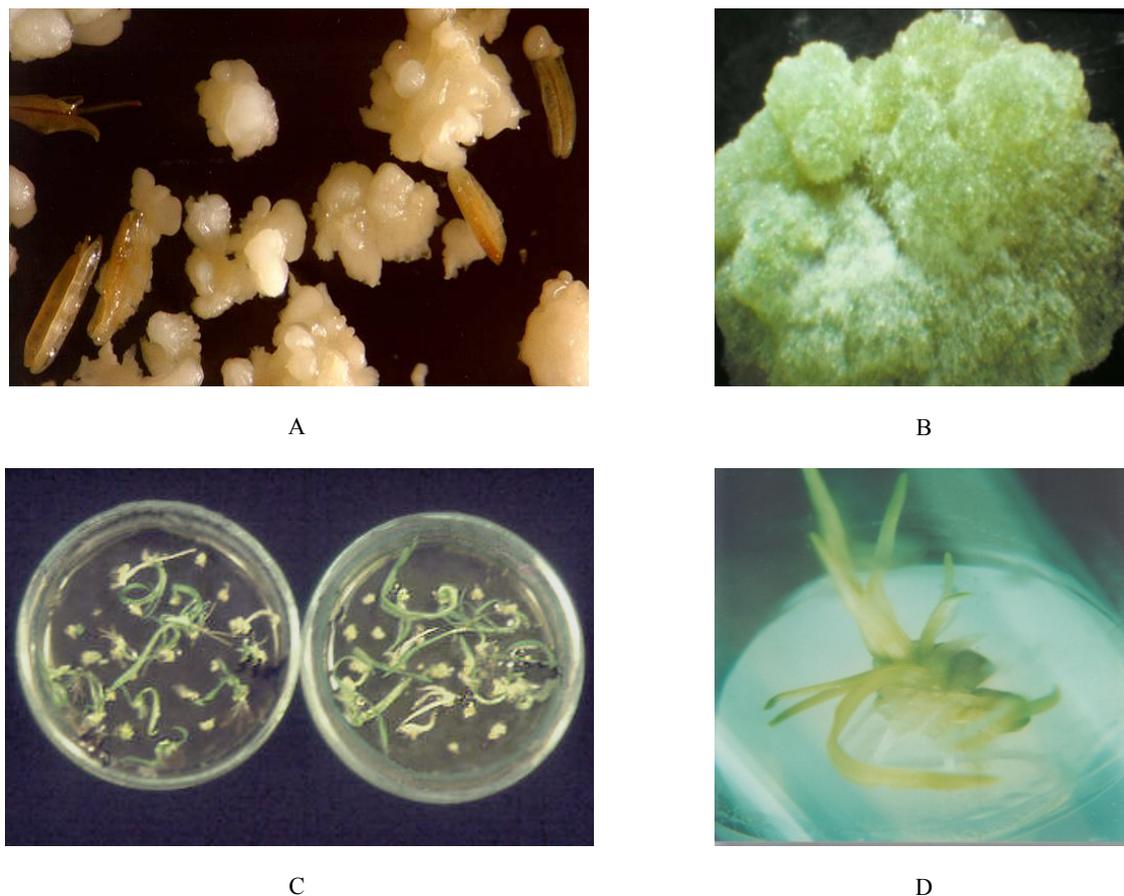


Figure 1 – The different steps from androgenic structure, embryoid induction to maturity. Embryoid (A) and morphogenic callus induction, x40 (B), green plant regeneration in N6 medium (C), haploid plant-regenerant (D)

In these experiments, the ratio of albino/green plants produced was very high (in particular, for hybrid BG 1-4/98 - 79.76%, for genotype BG 367 - 100%). Wietholter et al (2008) whereas other researchers (Cistue et al, 2004) also reported 100% ratio of albino/green plants in some barley cultivars [6, 7]. On the other hand, in embryogenesis and regeneration frequency there were significant differences between embryoids plated on N6 culture medium and embryoids cultivated on B5 medium. A decrease of ammonium nitrate with an increase in nitrate as in B5 medium composition have been identified as important factors in improving response. The embryogenesis frequency and respectively regeneration capacity in B5 culture medium were high for wheat hybrids BG 1-3/98 ($3,53 \pm 0,08\%$), BG 367 ($2,38 \pm 0,13\%$), BG 6-7/97 ($3,33 \pm 0,02\%$). However, some wheat genotypes, which anthers were cultivated in N6 medium, produced more (at 2-2.5 times more) embryoids, androgenic structure and green plantlets compared to

culture medium B5 ones (table 1, 2). The composition N6 medium including 100 mg/l myo-inositol, 9% (w/v) sucrose, 1 mg/l 2.4-D is optimal for production haploid plants in anther culture. 2.4-D is a synthetically occurring plant hormone that has auxin activity and support the growth of wheat callus culture in the presence of kinetin. S.Turasheva et al (2006) found that the presence of 2.4-D in culture media led to callus initiation and supported suspension culture growth in wheat. [8]. Furthermore, auxin 2.4-D induce embryogenesis in anther and microspore culture of wheat. With 2.4-D the induction response was not usually significantly different from controls but a significantly higher number of green plants were produced in wheat anther culture.

The dispersion analysis showed that influence of genotype on induction of morphogenesis and regeneration in spring wheat anther culture more (43.75%) than impact of culture medium factor (20.24%). On the other hand, influence of culture medium on development embryos and plants in

anther culture of spring soft wheat with Lr-gene (in particular, genotypes BG -L 9, BG -L 3) more than impact of genotype factor - 0,34% and 83,65% respectively (table 3).

Table 3 – The dispersion analysis influence of genotype (A) and culture medium (B) induction of morphogenesis and regeneration in wheat anther culture (two-factor experiment)

Factor	Degree of freedom	The amount of standard deviation	Dispersion	The contribution of impact, %	P fact.	P table. (0,99)
Spring soft wheat						
A-genotype	3	380,41	126,8	43,75	1196,19	29,46
B-culture medium	3	175,93	58,64	20,24	553,22	29,46
AB	9	300,11	33,35	34,52	314,56	
Spring soft wheat with Lr-gene						
A-genotype	1	0,81	0,81	0,34	11,75	
B-culture medium	3	201,43	67,14	83,65	978,46	
AB	3	34,6	11,53	14,37	168,06	

Probably, the response of parental lines in anther culture influenced the response of progeny combination. In the first case, there is a positive correlation between the anther response of the parents and the response of F₁ hybrid. However independent of culture medium composition a ratio of doubled haploid/haploid was 4.57: 1 and a ratio of haploid/aneuploid was 18,5:1.

Conclusion

In anther culture of wheat the high yielding donor plants produced more embryoids and green fertile plants than the middle yielding plants. Furthermore, for most donor hybrid plants influence of genotype on induction of morphogenesis and regeneration in anther culture more (43.75%) than impact of culture medium factor (20.24%). There is a positive correlation between the yielding capacity of the lines and the anther response. The optimal culture medium for production of wheat doubled haploids by anther culture is N6 culture medium including 100 mg/l myo-inositol, 9% (w/v) sucrose and 1 mg/l 2.4-D.

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