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An efficient method for the regeneration of wheat (*Triticum aestivum*) from anther cultures

by

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Abstract

An efficient procedure to obtain high yield of green wheat plantlets from anther culture is presented. The technique was applied to 4 spring genotypes and 2 winter genotypes, using a modified potato-2 medium [CHUANG *et al.*, 1978], supplemented with glutamine, with sucrose being pretreated with activated charcoal, and agarose replacing agar.

The total average percentage of anthers yielding at least one callus increased from 9.1 % (with the original potato-2 medium) to 21.6 % with our modified medium, while the total average percentage of regenerated green plants raised from 2.5 % (50 green plants from 2,040 anthers) to 6.9 % (369 green plants from 5,350 anthers).

Sucrose pretreatment of excised anthers greatly increased the rate of callus induction for 2 winter genotypes (F1 hybrids).

1. Introduction

In some plant species, haploids are easily produced from anther cultures. However, for most genera, including grasses, the rate of successful haploid production is low [PICARD *et al.*, 1978; MIAO *et al.*, 1978; BRETTELL *et al.*, 1981; BULLOCK *et al.*, 1982; FOROUGHI-WEHR *et al.*, 1982; GENOVESI and COLLINS, 1982; RINES, 1983].

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In wheat, breeding of doubled-haploids is currently limited by the low frequency of plantlet production per anther, specially in elite breeding lines [BULLOCK *et al.*, 1982; PICARD and DE BUYSER, 1977], thus making it difficult to be used effectively by plant breeders [GRIFFING, 1975].

Potato medium was much more effective in pollen callus induction than MS or Maiji 1 medium [Research Group 301, 1976], and has been widely used in many laboratories for anther cultures of wheat, barley and rye [WENZEL *et al.*, 1977; DE BUYSER and HENRY, 1979, 1980; SCHAEFFER *et al.*, 1979; SUNDERLAND *et al.*, 1979; BULLOCK *et al.*, 1982].

CHUANG *et al.* [1978] designed a new potato medium named potato-2 medium. The yield of pollen calli with potato-2 medium increased twofold as compared to medium N6 [CHU, 1978] or Maiji 1. Potato-2 medium has been gradually adopted by many laboratories [OUYANG, 1986].

Our paper deals with a modified medium, showing greater efficiency in regenerating green plantlets than the original potato-2 medium for 6 wheat genotypes.

2. Material and methods

2.1. MATERIAL

Six genotypes of field-grown wheat (*Triticum aestivum*) were used as anther donors, i.e. spring genotypes Sabine, Line 7, Atys and Echo, and winter genotypes F1 hybrid [(Zemon × V.P. M1) Gema] × [(Zemon × Mex.50-B21) Gema] H11972, from the «Station d'Amélioration des Plantes, Gembloux - Belgium» and N.R.P.B. 824997.H.1986.F1 of NICKERSON (W1 and W2 respectively).

2.2. METHODS

Donor plants were sown in mid-November for winter genotypes and late-April for spring genotypes. No chemical treatment was applied in the test plots during the growing period of donor plants.

Preliminary tests were carried out to show possible variations between pollen callus formation capacities of main spike or branched spikes of the same plant. In further experiments only the main spikes were used as donors.

We used anthers containing the mid- or late- uninucleate microspores, as described by HE and OUYANG [1984]. Anther stage was defined according to that of the majority of the pollen. Young spikes were disinfected with 0.1 % mercuric chloride for 8 min followed by 4 washings in sterile distilled water [CHUANG *et al.*, 1978]. After disinfection, intact anthers were immediately removed from the spikes and placed in 9 cm diameter Petri dishes (about 120 anthers per dish) containing 30 ml of solidified medium.

In some experiments, excised disinfected anthers were dipped for 1-2 hours prior to inoculation in a 0.8 M sucrose solution prepared as follows: sucrose (273 g) was dissolved in 1 l distilled water in which 30 g activated charcoal was added; the preparation was then autoclaved at 120°C for 20 min, and filtered; the filtered solution was autoclaved again at 110°C for 20 min, and used after cooling.

The potato extract and the original potato-2 medium were prepared as described by CHUANG *et al.* [1978]. The detailed composition of the potato-2 medium is as follows: 10% aqueous potato extract, 10^{-4}M Fe.EDTA, 1 mg/l thiamine HCl, 1.5 mg/l 2,4-D, 0.5 mg/l kinetin, 1000 mg/l KNO_3, 100 mg/l $(NH_4)_2SO_4$, 100 mg/l Ca $(NO_3)_2.4H_2O$, 125 mg/l $MgSO_4.7H_2O$, 200 mg/l KH_2PO_4, 35 mg/l KCl, 9-10% sucrose, 0.55-0.7% agar.

2.2.1. *Preparation of A medium*

The modified potato-2 medium (A medium) contained the components of the original medium, except for being supplemented with 200 mg/l glutamine, and semi-solidified with agarose (type VII, 5 g/l) instead of agar. Also sucrose, prepared as a 20% aqueous solution, was autoclaved at 120°C for 20 min in the presence of 2% activated charcoal; it was then filtered to remove the charcoal, and added to make 9% in the final A medium.

The components, to make 1 litre of medium (except for the potato extract), were dissolved in 600 ml distilled water and sterilized by ultrafiltration (0.2 μm filter).

Potato extract was diluted into 400 ml and autoclaved with agarose at 110°C for 20 min; its temperature was lowered to about 50°C before mixing with the above preparation to make 1 litre of A medium.

2.2.2. *Culture conditions*

Callus induction was carried out in the dark, or in dim light, at 27°C ± 1°C. Plant regeneration was obtained at 26°C under 1500 lux and a photoperiod of 12 hours.

2.2.3. *Culture temperature and pollen formation*

In order to establish the optimal temperature for callus induction and green plant regeneration, anthers were placed comparatively at 25°, 26°, 27°, 28°, 30°, 32°C. All treatments involved 3 replicates of 120 anthers.

2.2.4. *Regeneration of plantlets*

Calli, about 1 mm, were transferred onto a regeneration medium consisting of MS medium [MURASHIGE and SKOOG, 1962] with the macroelements at half concentration, naphtaleneacetic acid (NAA) at 0.5 mg/l, kinetin at 0.5 mg/l and agar (Difco Bacto agar) at 7.5 g/l.

When reaching 3 cm long, regenerated rooted or rootless plantlets were removed from the regeneration medium, and transferred into test tubes containing a root-inducing liquid medium [SCHAEFFER *et al.*, 1984], aerated by permanent shaking at 80-90 rpm in a gyrotary shaker, with a photoperiod of 16 hours light at 1500 lux. Plantlets were maintained in this medium until they developed sufficient roots for potting.

Cytological analysis of microspore embryogenesis during *in vitro* culture was based on the method described by HENRY *et al.* [1984], modified as follows. After 0, 6, 8, 10, 12, 16 days of culture, 8 randomly chosen viable anthers were removed from each of 3 Petri dishes per treatment, and fixed in acetic acid—alcohol (1:3) for observation.

For analysis of the percentage of anthers containing viable microspores, about 50 anthers were used for each treatment. In order to determine the developmental stage and the percentage of viable microspores, a sampling of 200 microspores taken from each anther (or from a mixture of 4 anthers) were observed. Microspores were considered to be alive when their nuclei and cytoplasm stained well in acetic-carmin.

Cytological analysis of regenerated green plants was performed according to the technique of HUCORNE [1987].

3. Results and discussion

3.1. EFFECT OF CULTURE MEDIUM

Comparison of A medium and potato-2 medium is shown in Table I. Pollen callus induction on A medium was 2-3 fold higher than that on potato-2 medium, with all genotypes used.

The enhanced frequency of green shoots differentiation was mainly due to the increase in responsive anthers frequencies on A medium.

The superiority of medium A over the original potato-2 medium may result from sucrose pretreatment with activated charcoal.

Charcoal might have absorbed 5'-hydroxymethylfurfural, a growth inhibitor produced by sucrose degradation during autoclave sterilization [WEATHERHEAD *et al.*, 1978]. Also, as suggested by BOULAY [1979], charcoal might bring some components favourable to growth and development of the explants, such as monophenylamines.

However, agarose may have played a favourable role as well, since agar is not the most suitable agent for anther culture [JOHANSSON, 1986].

Table I. — Comparison between A medium and Potato-2 medium.

Anther pretreatment	Genotype	Number of total cultured anthers		Number of responsive anthers		Number of responsive anthers/100 cultured anthers		Number of the total calli obtained		Number of calli/responsive anther		Number of regenerated plants		Number of regenerated plants/100 cultured anthers		Number of regenerated green plants		Number of regenerated green plants/100 cultured anthers	
		A (1)	P-2	A	P-2	A	P-2	A	P-2	A	P-2	A	P-2	A	P-2	A	P-2	A	P-2
No	Sabine	1,220	390	336	54	27.5	13.8	564	90	1.7	1.7	457	51	37.5	13.0	89	10	7.2	2.6
	Line 7	720	320	153	38	21.2	11.9	235	71	1.5	1.9	184	42	25.6	13.1	66	13	9.2	4.0
	Atys	770	340	174	25	22.6	7.4	248	47	1.4	1.9	199	30	25.8	8.8	59	10	7.7	2.9
	Echo	730	320	95	22	13.0	6.8	237	53	2.5	2.4	235	37	32.2	11.6	44	6	6.0	1.9
Pretreated with 0.8 M sucrose solution (3)	W1 (2)	1,270	310	357	31	28.1	10.0	875	94	2.5	3.0	518	50	40.8	16.1	90	9	7.1	2.9
	W2 (2)	640	360	39	16	6.1	4.4	69	24	1.8	1.5	60	13	9.4	3.6	21	2	3.3	0.6

(1) A = A medium; P-2 = Potato-2 medium.
For the composition of these media see Material and methods.

(2) W1 = hybrid F1: [(Zemon × V.P. M1) Gema] × [(Zemon × Mex. 50-B21) Gema] H11972.
W2 = N.R.P.B. 824997.H.1986.F1 of NICKERSON.

(3) Sucrose solution was pretreated with activated charcoal (see Material and methods).

3.2. EFFECT OF SUCROSE PRETREATMENT

WANG *et al.* [1981] treated excised wheat anthers with 0.8 M sucrose prior to culture. Their results showed that this treatment significantly increased the induction frequency of pollen calli. In our experiments, pretreatment of excised anthers with 0.8 M sucrose (pretreated with activated charcoal) had no effect with four homozygotic spring genotypes. A favourable effect was obtained, however, with hybrid winter genotypes, which showed an increase of the rate of callus induction raising from 16.1 % (without sucrose treatment) to 28.9 % (with sucrose treatment) for genotype W1, and from 3.8 % to 6.3 % for genotype W2, while the average percentage of regenerated green plantlets raised from 3.9 % to 6.8 % for W1 and from 1.9 % to 3.8 % for W2 (Table II).

Cytological study of genotype W1 (Table III), showed that sucrose pretreatment of excised anthers cultured on A medium increased the percentage of anthers containing living microspores. No modifications of the survival rate of microspores, and of the average number of nuclei per surviving microspore, were observed.

PAN *et al.* [1983] showed that early development of pollen was related to the viability of anther wall tissue; growth conditions and genotype of anthers donor plant may play a role in microspore callus induction, by affecting (or controlling) the activities of anther wall tissue [OUYANG, 1986].

In our tests, the increased frequency of anthers containing viable microspores, when pretreated with 0.8 M sucrose, may be linked either to heterozygotic or winter genotypes.

3.3. EFFECT OF TEMPERATURE

For all 6 genotypes we have studied, culture temperatures of 26°C to 28°C were favourable both for callus induction and green plantlets regeneration capacity. Typical data for W1 and Sabine are presented in figure 1. Increasing temperature for callus induction above 28°C did not modify significantly the frequencies of callus formation, but suppressed the capacity for green plant differentiation.

Our results are in general agreement with those of Research Group 301 [1977], showing that, although pollen callus yield increased with temperature, yet the capacity of calli to differentiate into green plantlets decreased accordingly. The studies of OUYANG *et al.* [1983] also indicated that the temperatures suitable for wheat anther culture ranged from 26°C to 30°C, according to the genotype of anther donor plants.

Table II. — Effect of sucrose pretreatment on callus induction and green plant regeneration frequencies(1).

Genotype	Pretreatment with 0.8 M sucrose	Cultured anthers	Responsive anthers	Responsive anthers / 100 cultured anthers	Number of			Green plants / 100 cultured anthers
					Calli or embryos observed	Green plants	Albino plants	
Sabine	without	420	117	27.8	172	30	169	7.1
	with	370	101	27.3	219	28	130	7.5
Line 7	without	400	81	20.2	139	35	55	8.8
	with	320	72	22.5	96	31	63	9.7
Atys	without	460	111	24.1	159	33	91	7.2
	with	310	63	20.3	89	26	49	8.4
Echo	without	420	51	12.1	147	26	108	6.2
	with	310	44	14.2	90	18	83	5.8
W1	without	380	61	16.1	116	15	34	3.9
	with	380	110	28.9	231	26	119	6.8
W2	without	160	6	3.8	9	2	8	1.3
	with	160	10	6.3	18	6	10	3.8

(1) See table I for legend.

Table III. — Pollen evolution *in vitro* with or without pretreatment with 0.8 M sucrose (Genotype W1 medium A)(1).

Observation	Pretreatment with 0.8 M sucrose	Age of culture (days)					
		0	6	8	10	12	16
% of anthers containing living microspore	with	100	99	72	68	49	38
	without	100	95	51	37	25	19
% of living microspore in living anthers	with	98.0	54.2	18.0	9.1	3.8	1.1
	without	98.0	32.0	11.3	4.5	2.1	0.9
Average number of nuclei per living microspore	with	1	1.8	2.6	4	8	> 8
	without	1	1.6	3.0	4	8	> 8

(1) See table I.

3.4. EFFECT OF DONOR SPIKE

In our hands, frequencies of callus formation in anthers from the main spikes were always higher than those of the branch spike for all 5 genotypes used (Figure 2).

3.5. EFFECT OF INOSITOL

Preliminary experiments with genotype Sabine (Table IV) indicated that addition of inositol (100 mg/l) in our callus induction medium increased both the number of calli per responsive anther and the number of green plants.

3.6. NUCLEAR STATE OF REGENERANTS

Cytological studies of 409 green plants regenerated from anthers showed about 80 % haploids, the dihaploidization of which is currently under investigation (Table V).

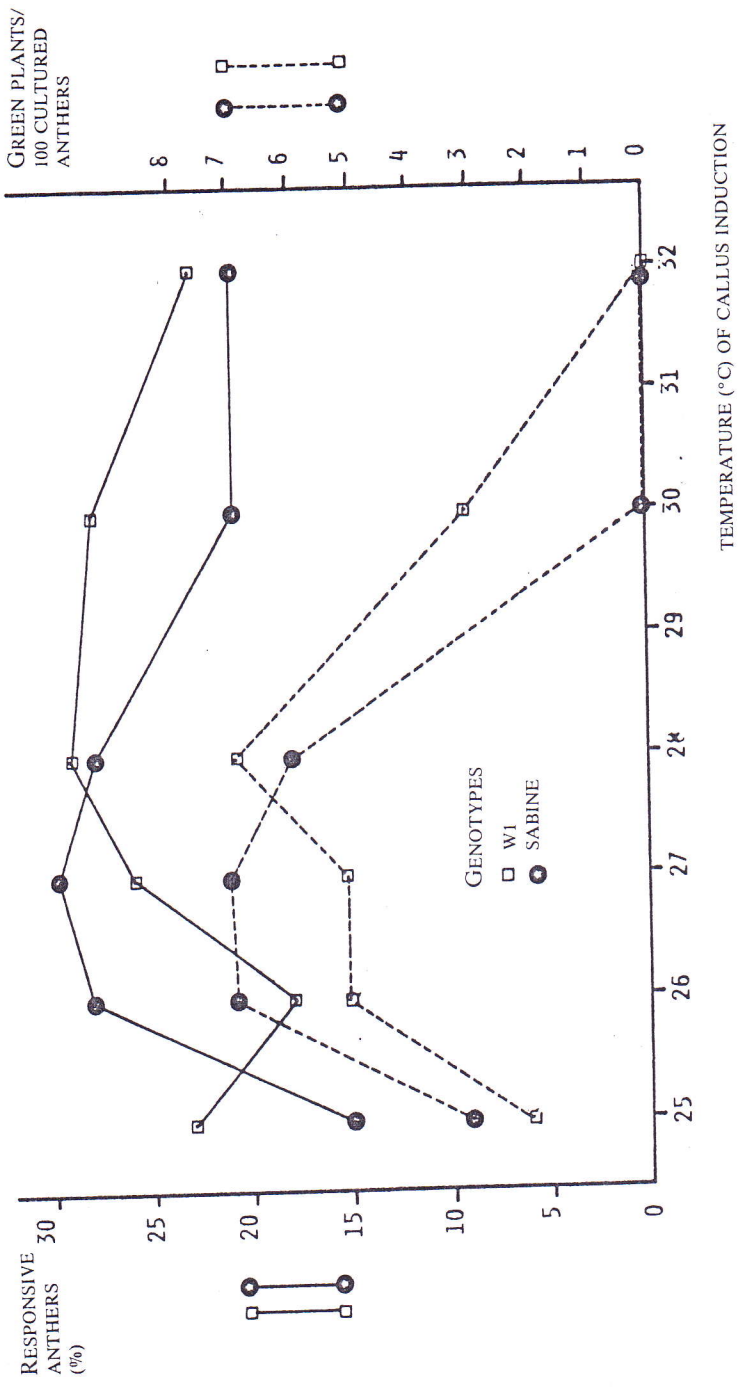

Figure 1. — Relation between anther response, green plants differentiation, and temperature of callus induction.

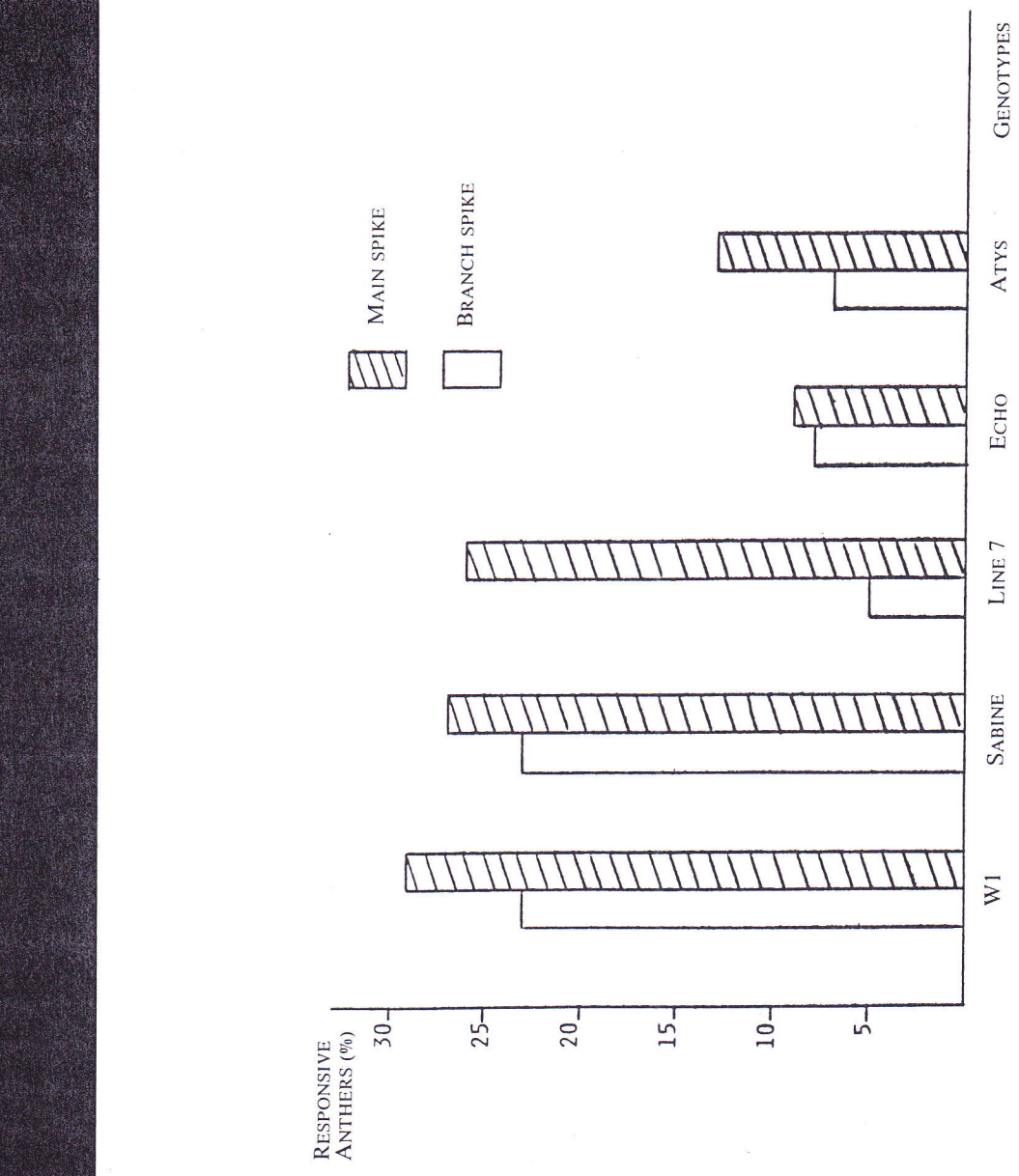

Figure 2. — Frequency of responsive anthers (callus formation) between main spike and branch spike containing pollen at the same developmental stage.

Table IV. — Effect of inositol on the regeneration of green plants from Sabine anthers.

Medium	Cultured anthers	Responsive anthers	Calli or embryos observed	Calli/responsive anther	Green plants	Albino plants	Green plant/100 cultured anthers
Medium A(1)	120	36	61	1.7	7	29	5.8
Medium A + 100 g inositol/l	120	31	96	3.1	12	15	10.0

(1) See table I.

Table V. — Cytological analysis of regenerated green plants.

Genotype	Total number of regenerated green plants	Haploids	Diploids	Aneuploids	Haploids/100 regenerated green plants
Sabine	97	74	14	9	76.3
W1 (1)	99	93	3	3	93.9
Line 7	79	64	9	6	81.0
Atys	63	45	15	3	71.4
Echo	50	43	1	6	86.0
W2 (1)	21	19	0	2	90.5
Total	409	338	42	29	82.6

(1) See table I.

Figure 2. — Frequency of responsive anthers (callus formation) between main spike and branch spike containing pollen at the same developmental stage.

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Résumé

Une méthode efficace de régénération du froment de printemps (Triticum aestivum) *à partir de cultures d'anthères*

La technique de culture d'anthères décrite, appliquée à deux génotypes de froment d'hiver et à quatre génotypes de froment de printemps, a permis d'obtenir un taux important de plantes chlorophylliennes.

Le milieu utilisé est dérivé du milieu potato-2 de CHUANG *et al.* [1978] par addition de glutamine, utilisation de saccharose traité au charbon de bois activé et remplacement de l'agar par de l'agarose.

Le pourcentage total moyen d'anthères produisant au moins un cal est passé de 9,1 % (avec le milieu potato-2 original) à 21,6 % avec le milieu modifié, tandis que le pourcentage total moyen des plantes chlorophylliennes régénérées passait de 2,5 % (50 plantes vertes pour 2040 anthères) à 6,9 % (369 plantes vertes pour 5350 anthères).

Le prétraitement au saccharose des anthères excisées a augmenté considérablement le taux de callogenèse pour les génotypes de froment d'hiver (hybrides F1).

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