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Improved anther culture method for obtaining direct regeneration in wheat (*Triticum aestivum* L.)

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ABSTRACT

Anther culture response of two wheat cultivars was tested with various barley-starch and maltose containing media. A considerable increase in direct plant regeneration (*i.e.* without transfer to a regeneration media) was obtained when medium was supplemented with silver nitrate. Up to 18 haploid plants per 100 plated anthers were directly produced. By using a single-step procedure, decreased costs in wheat haploid production can be effectively achieved.

Key words: Androgenesis, Bread wheat, Silver nitrate, Starch.

INTRODUCTION

Much effort has been put into developing anther culture techniques for cereals. Genotype and growth environment of the donor plants, developmental stage of the microspores, culture medium, and incubation conditions have been identified as critical variables in *in vitro* culture of wheat anthers (PICARD *et al.*, 1990). Despite considerable progress, current techniques are not yet suitable for broad application in wheat breeding programs. Wide application of the technique is largely dependent on increasing the level of haploid induction across genotypes while simultaneously decreasing procedural costs.

Conventional anther culture methods employ a two-step procedure in which embryoid induction and plantlet development occur in separate operations (DUNWELL, 1985). A single-step approach, in which plantlets are regenerated directly from microspores, would save time and materials if frequencies comparable to those of the two-step method could be attained. In barley anther culture, medium gelatinized with barley-starch instead of agar promotes the formation of embryoids which, occasionally, develop into plantlets (SORVARI, 1986a, 1986b).

In this study, different barley-starch media were tested for embryoid induction and development in wheat anther culture, and we report an improved procedure for obtaining a high frequency of direct regeneration of haploid plants.

MATERIALS AND METHODS

The spring wheat genotypes Veery's and Hodhod were used. Plants were grown, and anthers cultured as previously described (LASHERMES *et al.* 1991). The basal medium used was a modified MS medium (LASHERMES *et al.*, 1991) containing 750 mg/l glutamine and only 165 mg/l NH_4NO_3. Media for pollen embryoid induction were supplemented with 0.5 mg/l 2,4-D, and contained 30 g/l maltose and 60 g/l barley-starch. Barley-strach (Suomen Sokeri Oy, Finland) was mixed in cold distilled water and autoclaved. Cultures were incubated at 27°C in the dark. After 21 and 28 days, pollen embryoids and plantlets were counted. Embryoids which did not develop into plantlets, were transferred for regeneration to a medium consisting of basal medium with 34.2 g/l sucrose supplemented with 1 mg/l IAA, and cultured at 25°C under cool white fluorescent lights at 1500 lux for 16 h/day. Regenerated green plantlets were transferred to test tubes containing basal medium with 20 g/l sucrose and 0.5 mg/l IAA. Plants were ultimately transferred to soil and hardened gradually in the greenhouse.

The barley-starch gelatinized media are rather soft, and anther cultures tend to sink into the medium. An experiment was made to compare the effects of different solidifying or floating agents. Cultures were performed on starch media in which 6g/l agarose (Sigma type I), or polyester nets (mesh opening: 500 µm) laid on the nutrient media, were added. A double-layer medium was also tested in which the starch medium was overlaid with a Ficoll (Sigma type 400,20%) containing medium.

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A second experiment was made to evaluate the effect of silver nitrate (5 mg/l) and activated charcoal (Sigma, 10 g/l) on embryoid induction and development. A minimum of 600 anthers were cultured per treatment. Anthers from the same spike were randomly distributed among different media within each experiment.

For cytological analysis, root tips were collected from plantlets, pretreated with ice water for 16 h and then fixed in 3 parts of 95% ethanol: 1 part acetic acid (v/v). After treatment by a solution of 2% ferrous ammonium sulfate for a few hours, roots were stained in acetic carmine and squashed in a drop of 45% acetic acid for chromosome counts.

RESULTS AND DISCUSSION

The physical matrix of the barley-starch medium affected both embryoid induction and plant regeneration (Table 1). The use of polyester nets did not promote androgenesis. In contrast, the presence of agarose as solidifying agent increased embryoid production up to 149 embryoids per 100 anthers. Anthers were observed in which as many as 200 microspores began developing. Only a small fraction developed into embryoids and their regeneration ability was severely limited. Combined gelling effects of both agarose and barley-starch might have been too high so that a sufficient nutrient uptake was not guaranteed throughout the complete incubation period. In the Ficoll-supplemented medium, anthers floated on the surface of the medium. Embryoid production was lower due to a reduced number of responding anthers. However, most embryos had a dense globular structure and germinated into green plantlets on the induction medium within a few days. The double-layer starch/Ficoll medium resulted in a high proportion of directly generated plant, and in a good regeneration ability of the embryoids transferred to the regeneration medium.

The ratio of green to albino plants differed greatly between the various media. The number of albino plants was notably reduced in the Ficoll medium. As mentioned in previous reports (KAO, 1981; ZHOU and KONZAK, 1989), the embryoids produced in Ficoll-supplemented medium are released on the medium and float free of the dehisced anther. Therefore, they might have better access to nutrients and enhanced aeration.

The most remarkable feature in this experiment was the relatively high frequency of direct plantlets regeneration. As reported for barley (SORVARI, 1986a, 1986b), the use of barley-starch was found to be beneficial for the direct development of plantlets. Starch can be hydrolyzed in the presence of α-and/or β-amylase (SORVARI and SCHIEDER, 1987). However, the role of starch as an energy and carbon source in the medium is unknown. The osmotic potential of the medium plays an important role in microspore development (KAO, 1981), and starch may stabilize the medium's osmolality (KUHLMANN and FOROUGHI-WEHR, 1989).

Chromosome counts of roots tips indicated that the regenerated plants were predominantly haploid. Of 150 generated green plants 73% were found haploid ($n = 21$). The production of spontaneously doubled haploids (24 plants) was not medium specific (data not shown).

Presence of silver nitrate or activated charcoal affected anther culture responses of the two genotypes tested (Table 2). Numbers of anthers responding and, consequently, of embryoids and plants produced were enhanced when anthers were

TABLE 1

Effect of gelling agents on induction frequency and development of embryoids from wheat anther (cultivar Veery's') in media containing barley-starch*

Medium		Percent responding anthers**	Number of embryoids per 100 anthers	Number of plants produced per 100 anthers			
Agent	Degree of gelification			Direct generation	After transfer to a regeneration medium	Total	
						albino	green
Control	Semi-solid	21.1a	72.3b	4.1b	20.7a	14.6a	10.2a
Net	Semi-solid	17.4ab	58.1c	2.3b	20.1a	11.7a	10.7a
Agarose (6g/l)	Solid	18.5a	149.0a	0.5c	17.1a	6.3b	11.3a
Ficoll (20%)	Liquid	13.9b	40.5d	12.4a	2.0b	3.7c	10.7a

* A minimum of 600 anthers were cultured per treatment.
** Percentages followed by the same letter are not significantly different at the 0.05 probability level as determinated by «t» test of arcsin transformed data.

TABLE 2

Effect of $AgNO_3$ and activated charcoal on wheat anther culture response in media containing barley-starch*

Genotype	Medium		Percent responding anthers**	Number of embryoids per 100 anthers	Number of plants per 100 anthers					
					Direct regeneration			After transfer		
	$AgNO_3$ (5mg/l)	Charcoal (1%)			albino	green	total	albino	green	total
Hodhod	+	+	14.0a	30.6a	2.3	6.0	8.3a	0.0	0.0	0.0c
	+	—	5.7bc	24.1b	0.0	6.3	6.3a	0.7	4.6	5.3a
	—	+	7.7b	13.5c	0.0	2.3	2.3b	0.8	1.2	2.0b
	—	—	5.0c	28.1b	1.1	2.2	3.3b	1.5	4.4	5.9a
Veery's'	+	+	7.9a	16.3c	0.0	4.6	4.6b	0.0	0.8	0.8d
	+	—	10.0a	66.3a	2.1	16.2	18.3a	1.7	11.7	13.4a
	—	+	7.1a	17.9c	0.0	0.8	0.8c	2.5	0.0	2.5c
	—	—	7.9a	37.5b	0.0	3.8	3.8b	1.3	5.8	7.1b

* A minimum of 600 anthers were cultured per treatment.
** Percentages followed by the same letter are not significantly different at the 0.05 probability level as determinated by «t» test of arcsin transformed data.

cultivated on media containing 5 mg/l silver nitrate. These effects were modulated by the presence of activated charcoal and the genotypes used. Plant production through direct regeneration was improved by 2-5 times, depending on the genotype.

Ag^+ is known to be a potent inhibitor of ethylene action in plants (BEYER, 1976), and may promote wheat pollen embryogenesis and direct generation by blocking the inhibitory effect of endogenous ethylene on the embryo (PURNHAUSER *et al.*, 1987). An optimum level of ethylene production is important for pollen embryogenesis as reported in barley (CHO and KASHA, 1989) and *Solanum carolinense* (REYNOLDS, 1987).

When cultivated on media containing charcoal, the number of responding anthers increased in cv. Hodhod, whereas it did not differ significantly in cv. Veery's (Table 2). Both genotypes evidenced a significant reduction in embryoid production. The only exception was cv. Hodhod when activated charcoal was combined with silver nitrate.

Activated charcoal has been reported to stimulate anther culture on agar medium by removing inhibitory substances present in the medium or originating from the anther (JOHANSON *et al.*, 1982). In this study, the addition of charcoal resulted in a negative influence which may be due to the binding ability of charcoal not only for inhibitory substances but also for promoting substance.

Evidence from this study indicates that wheat haploid can be effectively produced via anther culture using a single-step procedure (Figure 1). Up to 18 plants per 100 plated anthers from cv. Veery's' were directly produced using a barley-starch gelatinized medium complemented with silver nitrate. In addition, regenerated plantlets appeared as soon as 3-4 weeks after anther inoculation. By reducing the quantity of work and time, decreased costs in wheat haploid production can be achieved.

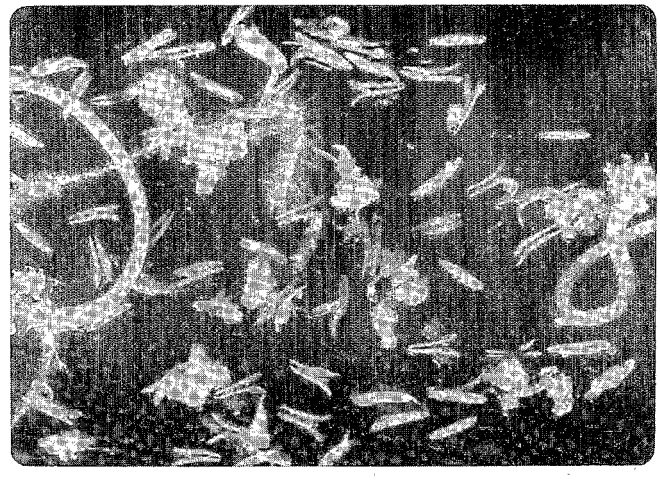

FIGURE 1 - Embryoid formation and direct plant regeneration from wheat anthers (cv Veery's) grown on silver nitrate barley-starch containing medium.

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