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Improved embryoid induction and green shoot regeneration from wheat anthers cultured in medium with maltose *

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Summary. Anthers from spring wheat (*Triticum aestivum* L.) genotypes, including six F_1 hybrids, were cultured in a modified liquid N6 medium containing either sucrose or maltose. In every case, use of maltose resulted in greater microspore callus induction and green shoot regeneration than culture in sucrose-containing medium. Induction in maltose medium also allowed green shoots to be recovered from crosses that showed only a poor response in other media and from two genotypes that did not respond to modified N6 medium with sucrose. Replacement of sucrose with maltose generally resulted in microspores having a more embryogenic mode of development in which distinct embryoids often formed. The most responsive genotype produced over 200 green shoots/100 anthers when cultured in medium with maltose.

Introduction

The use of anther culture to produce homozygous lines for wheat cultivar development has been widely advocated (Snape et al. 1986; de Buyser et al. 1987; Hu and Huang 1987). However, application of anther culture to wheat breeding is hampered by low callus induction and plant regeneration frequencies, as well as strong genotypic effects (Jones and Petolino 1987; Li et al. 1988; McGregor and McHughen 1990). Even with good callus induction, the haploid plants may be predominantly albino (Huang 1987; Ouyang et al. 1987; Zhou and Konzak 1989). Only when these problems are overcome can anther culture be used routinely as an adjunct to traditional wheat breeding methodology.

A number of factors critical to the response of cultured wheat anthers have been identified. These include anther donor plant growth environment (Jones and Petolino 1987; Ouyang et al. 1987; Bjornstad et al. 1989; Simmonds 1989), culture incubation temperature (Ouyang et al. 1987; Huang 1987; Simmonds 1989), and medium formulation (Marburger et al. 1987; Chu and Hill 1988; McGregor and McHughen 1990). The beneficial effect of liquid versus agar-solidified culture medium has also been established for wheat anther culture (Chu and Hill 1988; Zhou and Konzak 1989).

For other species, most notably barley, the particular carbon source in the induction medium can have a profound effect on anther culture response. Although sucrose is the most commonly used carbon source for all types of tissue culture, maltose and certain other carbohydrates were found to be superior for green shoot regeneration from barley anthers (Hunter 1987). This response was consistent for at least two cultivars. Maltose in conjunction with barley starch was also effective as a carbon source for androgenesis from several barley crosses (Kuhlmann and Foroughi-Wehr 1989).

For wheat anther culture, a recent report described the stimulatory effect of glucose on embryoid induction and plant regeneration (Chu et al. 1990). The effect was similar for a number of F_1 hybrids, but in most cases albino frequency remained high among the regenerants.

We have also examined the effect of various carbohydrates as a means of

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improving wheat anther culture. In this paper, we report a beneficial effect of maltose on callus and embryoid induction and green shoot regeneration from a range of wheat genotypes.

Materials and methods

Plant material. Anther donor plants were grown in a controlled environment, walk-in growth room with a 25/18 °C day/night temperature, a 16/8 h light/dark photoperiod, and an average light intensity at pot level of 330 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by fluorescent and incandescent lamps. Plants were grown in 15-cm diameter plastic pots containing Redi-Earth (W.R. Grace & Co.) and were watered every 2-3 days with a dilute nutrient solution (15:15:18/N:P:K, plus micronutrients).

The following spring wheat genotypes were used in the experiments reported here: cultivars HY320 and HY368, breeding line SWP2242, and F_1 hybrids 88058, 88067, 88070, 88072, 88084, 88085, 88116, 88247, 88267, 88281, 88292, 88298, 88305, 88312, 89038, 89044, 89054, 89062, and 89070. HY320 and a reselection therefrom, HY368, are registered cultivars. SWP2242 and all F_1 hybrids were developed by the Saskatchewan Wheat Pool wheat breeding program.

Anther culture. Tillers were harvested when the microspores were estimated to be at the mid- to late-uninucleate stage (He and Ouyang 1984). Tillers were sterilized by swabbing with 70% ethanol, then the flag leaf sheath was split and the spike was removed under aseptic conditions. One or two anthers were examined microscopically to verify microspore stage before culturing.

Anthers were cultured in autoclaved, liquid N6 medium (Chu 1978), modified according to Li et al. (1988) and referred to as MN6 medium. Standard MN6 medium contained 80 g/l sucrose (approximately 0.25 M) as a carbon source. For comparison with other carbohydrates, MN6 medium with 0.25 M sucrose was used. The disaccharides cellobiose, maltose, and trehalose, and the trisaccharides maltotriose and melezitose were tested, all at 0.25 M.

Media were dispensed into 10 x 35 mm plastic petri plates (Falcon) at 2 ml/plate. Anthers were plated at 20 (HY368, all crosses), 30 (SWP2242), or 40 (HY320) per plate and were incubated at 30 °C in the dark for microspore callus induction. The first calli appeared 3-4 weeks after culture initiation.

Plant regeneration. Plated anthers were examined every few days for the presence of calli. When calli reached a diameter of approximately 2 mm they were transferred to an agar-solidified regeneration medium with 1 mg/l indole-3-acetic acid and 1 mg/l kinetin (Schaeffer et al. 1979). Calli were then incubated at 26 °C at a light intensity of 15 $\mu E \cdot m^{-2} \cdot s^{-1}$ for shoot induction. Shoots were counted 3-4 weeks after transfer of calli to regeneration medium.

Results

Effect of various carbohydrates on callus induction and shoot regeneration in cultivar HY320

The first experiment was performed using cultivar HY320, which was found to be a responsive genotype in preliminary experiments as well as in an earlier study (Li et al. 1988).

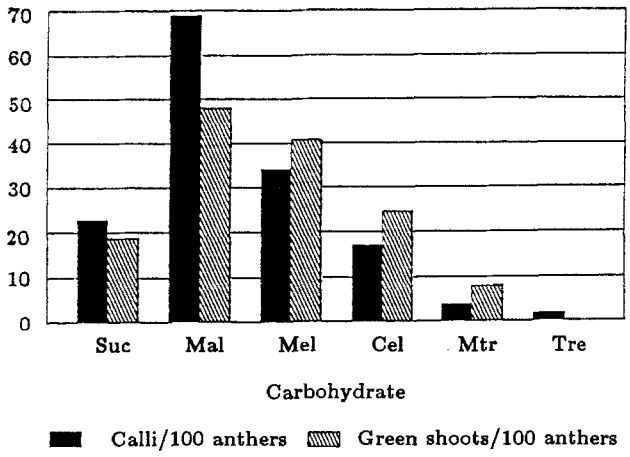

Figure 1. Callus induction and green shoot regeneration from anthers of HY320 cultured in MN6 medium with various carbohydrates. Suc - sucrose; Mal - maltose; Mel - melezitose; Cel - cellobiose; Mtr - maltotriose; Tre - trehalose. 240-280 anthers per treatment.

On MN6 induction medium with sucrose, HY320 produced 22.9 calli/100 anthers. One third of these calli formed green shoots when transferred to regeneration medium and less than 10% produced albinos. Calli from anthers cultured in medium with sucrose yielded 18.8 green shoots/100 anthers in this experiment.

Anthers of HY320 produced calli in media containing each of the other carbohydrates tested but the response was best in medium with maltose (Fig. 1). Callus induction was increased by 3-fold and the number of green shoots per 100 anthers more than doubled in comparison to sucrose. Albinos were produced from 20% of the calli. Thus culture on maltose medium led to an increase in both green and albino shoot regeneration in this initial experiment.

Effect of maltose on callus induction and shoot regeneration in F_1 hybrids

To determine if the beneficial effects of maltose observed for HY320 would be similar for other genotypes, anthers from six F_1 hybrids were cultured in MN6 medium with sucrose or maltose and their responses on the two media were compared. In every case, maltose was superior to sucrose for callus induction and green shoot regeneration (Table 1).

The response of certain F_1s was especially dramatic on maltose-containing medium. F_1 88267 underwent a more than 20-fold increase in the number of calli per 100 anthers compared to medium with

Table 1. Callus induction and green shoot regeneration from anthers of six F_1 hybrids cultured in MN6 medium with sucrose or maltose.

F_1		Calli/100 anthers	% Calli with green shoots	Green shoots/ 100 anthers
88247	Sucrose	4.0	17	0.7
	Maltose	15.0*	49	19.7**
88267	Sucrose	4.6	0	0.0
	Maltose	106.3**	25	76.7**
88292	Sucrose	15.3	30	18.3
	Maltose	37.7*	83	115.3**
88298	Sucrose	4.7	29	2.3
	Maltose	31.0**	19	17.0*
88305	Sucrose	14.0	33	14.7
	Maltose	34.3*	31	42.7*
88312	Sucrose	3.3	10	0.7
	Maltose	31.7**	14	13.3**

*,** Difference between sucrose and maltose significant according to t-test at p=0.10 and p=0.05, respectively. Data subjected to square root (x + 0.5) transformation before analysis. 280-300 anthers cultured per treatment.

sucrose (Table 1). Green shoot regeneration from 88267 calli was also high for cultures induced on maltose, whereas no green shoots were recovered from calli of this F_1 initiated on sucrose-containing medium. Albino frequency was 15% for calli of 88267 induced on sucrose and 31% for calli induced on maltose medium.

The greatest regeneration response among the crosses cultured in this experiment was seen for F_1 88292. This hybrid produced more than 100 green shoots/100 anthers (Table 1).

Subsequent studies identified at least two genotypes besides F_1 88292 with exceptionally high levels of green shoot regeneration from calli induced on maltose medium (Table 2). These genotypes also had low albino frequencies. The best regeneration response to date was seen for cultivar HY368 which produced as many as 210.7 green shoots/100 anthers.

Effect of maltose on the response of recalcitrant genotypes

The cultivars and F_1s cultured in the experiments described above belong to the CPS (Canada Prairie Spring) class of wheat, which is characterized as having medium to hard kernels, intermediate protein content, and relatively high yield. In general, CPS genotypes have a higher

Table 2. Green and albino shoot regeneration from anthers of three highly responsive genotypes cultured in MN6 medium with maltose.

Genotype	Calli with shoots (%) Green	Albino	Green shoots/100 anthers
HY368	76	18	210.7
F_1 88292	83	5	115.3
F_1 88281	72	16	112.5

Table 3. Callus induction and green shoot regeneration from anthers of SWP2242 cultured in MN6 medium with sucrose or maltose.

Experiment	Anthers cultured	Calli/100 anthers	% Calli with green shoots	Green shoots/ 100 anthers
1 Sucrose	300	0.0	--	0.0
1 Maltose	300	8.0	46	9.7
2 Maltose	640	16.4	49	21.4
3 Maltose	390	11.8	72	22.8

androgenetic response than members of the CWRS (Canada Western Red Spring) class, the high-protein, hard red spring wheats, under various culture conditions (unpublished observations). The use of maltose medium was therefore tested as a possible means of improving the response of recalcitrant CWRS genotypes.

CWRS line SWP2242 was cultured in the first of these experiments. As shown in Table 3, no calli were produced from anthers in MN6 medium with sucrose. However, by changing the carbohydrate to maltose, callus induction was achieved and green shoots were recovered. Close to 50% of the calli regenerated green shoots and only one callus developed albino shoots.

Subsequent experiments confirmed the beneficial effect of maltose on the induction and regeneration response of SWP2242 (Table 3). As many as 22.8 green shoots/100 anthers have been obtained from calli of this previously difficult genotype following culture in MN6 medium with maltose.

Anthers of twelve CWRS F_1 hybrids were also cultured in medium with maltose. Callus induction and green shoot regeneration occurred for eleven of these hybrids (Table 4), but the frequencies were generally lower than for CPS F_1s (Table 1). These results show that the low androgenetic response typical of CWRS genotypes is not entirely overcome by

Table 4. Callus induction and green shoot regeneration from anthers of twelve CWRS F_1 hybrids cultured in MN6 medium with maltose.

F_1	Anthers cultured	Calli/100 anthers	% Calli with green shoots	Green shoots/ 100 anthers
88058	320	8.4	52	9.1
88067	220	0.0	--	0.0
88070	200	14.5	24	7.0
88072	520	8.8	28	5.2
88084	1320	4.4	50	5.4
88085	660	7.7	63	8.9
88116	1040	3.8	46	4.0
89038	640	3.1	65	11.3
89044	400	8.8	66	24.8
89054	320	10.9	43	34.7
89062	540	1.5	13	0.7
89070	580	1.9	36	1.7

switching from sucrose to maltose in the induction medium. However, green shoot regeneration was at least comparable to that obtained when a sample of these F_1s was cultured on several other media (data not shown). Albino frequency was also low (0 to 18%) for all but one of these hybrids (88070; 45% albino) following culture in MN6 medium with maltose.

Effect of maltose on embryoid versus callus induction

When anthers were cultured in medium with sucrose, the typical response was the formation of microcalli which began to emerge from the anthers after about 4 weeks. These calli were sometimes embryogenic and formed shoots on regeneration medium. Often, however, the calli died or formed only roots.

In contrast, distinct embryoids often developed from anthers in maltose medium. Individual embryoids were most prevalent during the early part of the induction phase and quickly proliferated to form embryogenic calli. The embryoids and resulting calli had great potential for shoot regeneration and sometimes germinated while still in the induction medium. Multiple shoots were common from these structures.

Discussion

The results presented here clearly show that the carbon source used for wheat anther culture can have a significant effect on androgenetic response. Maltose was superior to sucrose both for callus induction and green shoot regeneration for a range of genotypes. The use of maltose also allowed green shoots to be recovered from genotypes (F_1 88267, SWP2242) which failed to produce shoots when anthers were cultured in medium with sucrose.

Carbon source has been implicated as a key factor in a number of tissue culture systems. There is increasing evidence that sucrose is most effective for supporting unorganized cell proliferation (Kinnersley and Henderson 1988) or the growth of organs and meristems (Pareddy and Greyson 1985, 1989), whereas other carbohydrates are more suitable for somatic embryogenesis or androgenesis. Embryogenesis from callus cultures may be stimulated by glycerol (e.g. *Citrus* spp.; Ben-Hayyim and Neumann 1983), maltose or malt extract (e.g. *Medicago sativa*; Strickland et al. 1987), or corn syrup, which consists primarily of glucose and maltose (e.g. *Daucus carota*; Kinnersley and Henderson 1988). High maltose corn syrup induced plantlet formation from *Nicotiana tabacum* anthers (Kinnersley and Henderson 1988) and glucose, cellobiose, maltose, trehalose, maltotriose, and melezitose were all more effective than sucrose for barley anther culture (Hunter 1987). Polysaccharides such as barley starch (Sorvari and Schieder 1987; Kuhlmann and Foroughi-Wehr 1989) and dextrin (Hunter 1987) also improved the response of barley anthers in culture. These effects of alternative carbohydrates all involve cell differentiation. We found that maltose as a carbon source for wheat anther culture resulted in a more organized mode of development, i.e. a tendency toward embryoid induction compared to primarily callus formation for cultures on sucrose.

While the effects of maltose and alternative carbohydrates have been documented in several tissue culture systems, the reason for their superiority to sucrose is not known. Other authors have indicated that maltose improves the osmotic stability of the culture medium compared to sucrose (Kuhlmann and Foroughi-Wehr 1989). However, since maltose may stimulate embryogenesis at low concentrations and is superior to sucrose when used at equal osmolarity (Strickland et al. 1987), its effect must be at least partly nutritional. The break-down products of maltose and sucrose differ, which may be a key to the advantage of maltose. It is also possible that maltose is broken down more slowly than sucrose, providing a readily metabolizable carbon source over a longer period of culture.

Maltose in the induction medium did not totally eliminate the genotypic effect that is so prevalent in wheat anther culture. Although induction and regeneration responses were increased for every genotype where a comparison with sucrose was made (Fig. 1; Tables 1 and 3), culture on maltose medium still allowed high and low responding genotypes to be identified. It may be possible to further modify the protocol to improve the response of the poorer responding material. For example, lower concentrations of maltose may be beneficial, as was the case for barley (Hunter 1987; Kuhlmann and Foroughi-Wehr 1989). A combination of carbohydrates, perhaps including glucose, might also be worth investigating (Chu et al. 1990).

The regeneration medium used here was a standard medium (Schaeffer et al. 1979) containing 3% sucrose. We are not certain if the addition of maltose to this medium would have a beneficial effect beyond that seen with maltose in the induction medium. However, it would appear this is not essential since green shoots were formed from as many as 70-80% of the calli or embryoids induced on maltose medium and transferred to the regeneration medium with sucrose (Table 2).

Three of the genotypes used in this study displayed an exceptionally high level of green shoot regeneration. Cultivar HY368 and CPS hybrids 88281 and 88292 yielded, on average, over 100 green shoots per 100 anthers cultured. Individual plates of anthers from these genotypes were significantly above these average responses. For example, the best plate of HY368 (containing 20 anthers) produced 91 green shoots, equivalent to 455 per 100 anthers.

Albinism is often a limiting factor in wheat anther culture, even when the overall induction and regeneration frequencies are high (Huang 1987; Ouyang et al 1987; Zhou and Konzak 1989). This was the case in much of our early work, especially with CWRS genotypes, where over 90% of the regenerated shoots were albino (unpublished observations). Culture of anthers on maltose medium sometimes increased albino shoot regeneration, for example with genotype HY320 and some of the CPS crosses (data not shown). However, for the difficult CWRS genotypes, maltose in the induction medium led to a shift from albino to predominantly green shoot regeneration. The use of maltose as a carbohydrate in the induction medium is the only factor we have been able to identify which consistently enhanced green versus albino shoot production from anther cultures of poorly responding genotypes such as SWP2242 (Table 3). This study has led us to use MN6 medium with maltose for the routine culture of anthers from all genotypes in our program.

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Note. While this manuscript was under review, a paper by D.I. Last and R.I.S. Brettell appeared (Plant Cell Rep 9: 14-16) describing the beneficial effect of maltose on embryo induction from anthers of four spring wheat cultivars. The highest response occurred for cultivar Orofen which yielded 50 embryos/100 anthers.

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