J. Appl. Genet. 38(4), 1997, pp. 381-392

Wheat anther culture as affected by various cultural changes and supplements

R.L. SIMONSON[1], P.S. BAENZIGER[2], V.D. GUSTAFSON[2]

[1]Terra International 600, Fourth St. Sioux City, Iowa, USA
[2]Department of Agronomy, University of Nebraska, Lincoln, NE, USA

Abstract. With the numerous improvements in cereal tissue and wheat anther culture, it is necessary to determine which of the improvements should be combined for optimal response. This study was conducted using one highly responsive cultivar of wheat (*Triticum aestivum* L. cv. Pavon 76) to test the effectiveness of pre-culture cold treatment (0 or 3-7 days at 5°C) of anthers, five initiation basal media, and various changes in Murashige-Skoog regeneration media. A cold pre-culture treatment was inhibitory for all initiation media for embryoid initiation. Of the initiation media, P1, 85D12, and N6 were similar for embryoid initiation (0.80 to 0.90 embryoids/anther) without a cold pre-culture treatment. Plant regeneration was improved by the addition of amino acids or glucose, increased sucrose concentration, filter sterilizing the medium, and altering plant growth regulator concentrations. P1 medium which is normally used for embryoid initiation was also beneficial for improving plant regeneration. Ethylene inhibitors were generally not beneficial.

Key words: anther culture, pre-culture cold treatment, silver nitrate, *Triticum aestivum* L.

Introduction

An optimized wheat (*Triticum aestivum* L.) anther culture system must have the potential to produce large numbers of microspore-derived doubled haploid plants which are required for doubled haploid wheat breeding. Culture improvements are continuously being made to increase the number of embryoids initiated from anthers and the number of plants regenerated from embryoids. It is necessary to determine which improvements can be combined to improve the overall system.

Received: July 1997.
Correspondence: P.S. BAENZIGER, Department of Agronomy, University of Nebraska, Lincoln, NE 68583-0915, USA.

Anthers are often exposed to a cold treatment before they are placed on embryoid initiation medium (PICARD, de BUYSER 1975, HENRY, de BUYSER 1981, SHIMADA 1981, WEI 1982, MARSOLAIS et al.1984, LIANG, MCHUGHEN 1987, LAZAR et al.1990). The cold treatment remains controversial as it is not always beneficial (LIANG et al. 1982, OUYANG 1986, CHU, HILL 1988).

Initiation and regeneration media have been modified to determine which constituents best promote haploid plant production. Potato 1 (P1) (Research Group 301, 1976) and Potato 2 (P2) (CHUANG et al. 1978) have been popular, although potato variation and its unknown extracted constituents are of concern (LIANG, MCHUGHEN 1987). Defined chemical component media have been proposed for their reproducibility. Several basal media for embryoid initiation and plant regeneration include Murashige and Skoog (MS) (MURASHIGE, SKOOG 1962), N6 (CHU 1978), modified N6 (MN6) (CHU, HILL 1988), and 85D12 (LIANG et al. 1987).

In addition to the basal medium, other medium constituents in wheat tissue culture have been modified or added including plant growth hormones and their inhibitors (PAPENFUSS, CARMEN 1987, HASSAWI et al. 1990), saccharide type and concentration (KELLY 1986, CARMEN et al. 1987b, BIDDINGTON et al. 1988, CHU et al. 1990, LAST, BRETTELL 1990, ORSHINSKY et al. 1990), amino acids (CARMEN et al. 1987b, ZHU et al. 1990), antibiotics (MATHIAS, BOYD 1986), osmoticums (MARBURGER et al. 1987, ZHOU et al. 1991), nitrogen sources (OLSEN 1987, FENG, OUYANG 1988), and gelling agents (SIMONSON, BAENZIGER 1992). Plant growth regulators used in plant regeneration media include 2,4-dichlorophenoxyacetic acid (2,4-D), napthaleneacetic acid (NAA), 6-(dimethyallyamino)-purine (2iP), kinetin, indole-3-acetic acid (IAA), benzylaminopurine (BAP), indole-3-butyric acid (IBA), gibberellic acid (GA3), combinations of the above, or media without plant growth regulators.

Adding ethylene inhibitors to culture medium improved wheat immature embryos response in culture (PURNHAUSER et al. 1987, QURESHI et al. 1989). Ethylene inhibitors have also been important in the tissue culture of diverse crops (CARMEN et al. 1987a, BIDDINGTON et al. 1988, PERL et al. 1988, CHI, PUA, 1989, VAIN et al. 1989). Ethylene inhibitors include silver nitrate ($AgNO_3$), silver thiosulfate ($Ag_2S_2O_3$), abscisic acid (ABA), abscisic acid methyl ester (MeABA) and acetylsalicylic acid (ASA). Embryogenesis is related to ethylene production (CHO, KASHA 1989, VAIN et al. 1989) and its production is often induced by auxin hormones (REYNOLDS, 1987) which are included in initiation media and reduced in regeneration media.

Sucrose concentrations in plant regeneration media have varied from 58 to 200 mM (CHU, HILL 1988). Several additions to anther culture media have

promoted embryoid inititation and plant regeneration including 10 mM glucose (KELLY 1986), 2% (v/v) polyethylene glycol (PEG) (THORN 1988), amino acids (CHU, HILL 1988) and filter sterilizing chemical constituents (CHU et al. 1990). Cefotaxime was observed to promote increased calli growth and shoot production when added to immature wheat embryo culture medium (MATHIAS, BOYD 1986).

The purpose of this study was to combine previously reported improvements to enhance our anther culture system; specifically determine the need for giving anthers a pre-culture cold treatment, and the effect of various basal media and other additions on embryoid initiation and plant regeneration.

Material and methods

Plants of spring wheat Pavon 76 were grown in 15 cm pots containing soil, in a growth chamber with a 12 h photoperiod (light intensity of 350 μmol s^{-1} m^{-2}) per 24 h day, and a temperature of 18°C day, 13°C night temperatures. After one month, the plants were moved to a second growth chamber with similar conditions except for a 16 h photoperiod per 24 h day to induce flowering. Plant tillers were harvested when they were in the boot stage and the top of the inflorescence was between the penultimate leaf and flag leaf. This coincided with the microspores being at the mid- to late-uninucleate stage. The microspore stages were intermittently checked using a light microscope and acetocarmine stain. Some tillers were given a cold treatment of 5°C for 3-7 days while the others were used directly for anther excision for embryoid initiation. The tillers were surface disinfected for 20 min. in 1.05% (v/v) sodium hypochlorite with periodic agitation. The anthers were aseptically excised and 30 were placed on each 60 × 15 mm Petri dish containing one of five liquid initiation media (described below) with 10% (w/v) Ficoll type 400 to increase viscosity and keep initiated embryoids buoyant in the liquid media. At least 300 anthers were plated for each cold treatment and initiation medium combination (2 cold treatments by 5 initiation media) for a total of 4,350 anthers plated. These dishes containing anthers were placed in a dark growth chamber with a temperature of 28°C for embryoid initiation.

The five initiation media were as follows. P1 contained 0.1 μM FeEDTA, 3.0 μM thiamine HCl, and 200 g potatoes per liter of medium. The potatoes were chopped, boiled and strained through four layers of cheese cloth. P1 with MS salts (P1/MS) contained 100 g potatoes for one liter of medium, prepared as above, with MS salts at one half their normal concentrations, 0.1 μM

FeEDTA, and 3.0 μM thiamine HCl. MS, N6 and 85D12 media also were used. Each medium except 85D12 contained 6.8 μM 2,4-D, 2.3 μM kinetin and 0.26 M sucrose. To medium 85D12, 10 μM p-chlorophenoxyacetic acid (pCPA), 4.7 μM kinetin and 0.22 μM sucrose was added (YUAN et al. 1990).

When embryoids and polyembryoids (hereafter included with embryoids) were 1.0-2.0 mm in diameter, (27 to 193 days after being placed on initiation media) they were randomly transferred to regeneration media to promote plant regeneration. Twelve MS based regeneration media (four plant growth regulator concentrations and three $AgNO_3$ concentrations) with 0.76% (w/v) Bacto-agar were tested (Table 2). Plant regeneration was induced in an incubator with a temperature of 24°C and a light intensity of 30 μmol s^{-1} m^{-2}.

A subsequent experiment to test additional regeneration media (Table 4) modified with plant growth hormones and ethylene inhibitors, amino acids, saccharide type and concentrations, and osmoticums used embryoids from experiments studying the effect of gelling agents on embryoid initiation (SIMONSON, BAENZIGER, in press). In this experiment, the regeneration media were compared to medium containing MS basal salts, 2.7 μM NAA, 2.3 μM kinetin, 58 mM sucrose and 0.76% Bacto-agar (identified in the first experiment) for their ability to promote plant regeneration.

Chi-square statistics were used to identify statistically significant differences ($P < 0.05$) among initiation and regeneration media. The statistics were done on the number of embryoids or plants in the indexes described below, adjusted for the number of anthers, or embryoids used:

1) embryoid-initiation frequency = number of embryoids transferred/number of anthers plated,

2) embryoid-regenerating frequency = number of embryoids producing green plants/number of embryoids transferred,

3) green-plant frequency = number of green plants produced/number of embryoids transferred.

Results and discussion

Embryoid initiation

Anthers without a cold treatment prior to plating produced significantly more embryoids than the anthers with a cold treatment for every initiation media. Averaged over five initiation media, anthers without a cold treatment had a significantly higher embryoid initiation frequency (0.620) than anthers with a cold treatment (0.408, Table 1). A cold pre-culture treatment is often

Table 1. Number of anthers, embryoids and the embryoid initiation frequency for each initiation medium and anther cold treatment

Initiation medium	Number of anthers	Number of embryoids	Embryoid initiation frequency		
No cold					
P1	450	408	0.907	a+	A++
85D12	300	255	0.850	a	A
N6	300	241	0.803	a	A
MS	360	155	0.431	b	A
P1/MS	360	38	0.106	c	A
Total	1770	1097	0.620		A
Cold treatment					
P1	600	460	0.767	a	B
85D12	600	460	0.767	a	B
N6	540	253	0.469	c	B
MS	540	94	0.174	d	B
P1/MS	540	30	0.056	e	B
Total	2580	1053	0.408		B

+ Entries followed by the same small letter are not significantly different ($P < 0.05$) within the same cold treatment as determined by Chi-square statistical tests.

++ Entries followed by the same capital letter are not significantly different ($P < 0.05$) between the same medium with a different cold treatment as determined by Chi-square statistical tests.

used in wheat anther culture although several studies have not observed the procedure to be beneficial (LIANG et al. 1982, OUYANG 1986, CHU, HILL 1988). One recent study observed the cold pre-culture treatment to promote an increase in the number of responding anthers and the number of embryoids initiated (LAZAR et al. 1990). However, Pavon 76 was not one of the cultivars used. Hence, the need for a cold treatment may be genotype dependent.

The embryoid initiation frequencies for anthers on the five initiation media were significantly different within or averaged over cold treatments. The ranking of the media according to embryoid initiation frequency of Pavon 76 was the same regardless of cold treatment (Table 1), though a cold treatment may be more efficient in statistically separating the media. This is an important finding because much of the previous literature using Pavon 76 included cold treatments. The media improvements made with Pavon 76 with a cold treatment should be applicable to media improvements for Pavon 76 without a cold treatment.

Anthers without a cold treatment on P1 initiation medium (0.907) had the highest embryoid initiation frequency, but was not significantly different from 85D12 (0.850) or N6 (0.803) media. N6 medium was successfully used in another study where 'Orofen' and 'Pitic 62' anthers resulted in embryoid initiation frequencies of 0.853 and 0.571, respectively (HUANG 1987). Anthers on MS and P1/MS media had the lowest embryoid initiation frequency. One reason for the poor performance of MS medium may be related to its NO_3^- to NH_4^+ ratio. MS medium has a NO_3^- to NH_4^+ ratio of 1.9 to one. 85D12 and N6 media have NO_3^- to NH_4^+ ratios of 4.7 and 4.0 to one, respectively. Embryoid initiation frequencies have been observed to increase when the NO_3^- to NH_4^+ ratio in the initiation medium was increased to 3-4 to 1 (FENG, OUYANG 1988). P1 and P1/MS medium are not defined medium, thus their NO_3^- and NH_4^+ concentrations are not known.

Plant regeneration

In the first regeneration experiment, several regeneration media were observed to promote plant regeneration. Embryoids on regeneration media containing 2.7 µM NAA and 2.3 µM kinetin with zero or 60 µM silver nitrate, and embryoids on media containing 5.4 µM NAA and 0.5 µM 2iP at any of the three silver nitrate concentrations, had statistically similar embryoid regenerating frequencies (ranging from 0.121 to 0.187, Table 2). The embryoids

Table 2. The embryoid regenerating frequency and green plant frequency on each regeneration medium

Plant growth regulator conc. (µM)	Silver nitrate conc. (µM)	Number of embryoids	Embryoid regenerating frequency		Green plant frequency	
2.7 NAA +2.3 kinetin	0	155	0.187	A[+]	0.355	A
2.7 NAA +2.3 kinetin	60	126	0.119	A	0.254	AB
2.7 NAA +2.3 kinetin	300	111	0.054	B	0.063	DE
5.4 NAA +0.5 2iP	0	157	0.121	A	0.223	B
5.4 NAA +0.5 2iP	60	154	0.143	A	0.227	B
5.4 NAA +0.5 2iP	300	153	0.150	A	0.189	BC
2.7 2,4-D	0	99	0.010	BC	0.010	F
2.7 2,4-D	60	99	0.020	BC	0.020	EF
2.7 2,4-D	300	113	0.026	BC	0.026	EF
2.7 2,4-D +2.3 kinetin	0	97	0.000	C	0.000	F
2.7 2,4-D +2.3 kinetin	60	98	0.041	B	0.071	DE
2.7 2,4-D +2.3 kinetin	300	96	0.052	B	0.093	CD

[+]Entries followed by the same letter within the same column are not significantly different ($P<0.05$) as determined by Chi-square statistical tests.

on the remaining media had significantly lower embryoid regenerating frequencies.

Silver nitrate had detrimental or no effects on regeneration media with high embryoid regenerating frequencies, but was able to improve a medium with low embryoid regenerating frequency. Silver nitrate had a detrimental effect on the embryoid regenerating frequency when 300 μM was added to media containing 2.7 μM NAA and 2.3 μM kinetin (Table 2). However, silver nitrate added to media with 2.7 μM 2,4-D and 2.3 μM kinetin increased the number of embryoids producing green plants (0.041 to 0.052), compared to the same medium without silver nitrate (0.000). Embryoids on this medium may have induced more ethylene production, which hinders plant regeneration and the effect was counteracted by $AgNO_3$ (PURNHAUSER et al. 1987). An optimum level of ethylene was observed to be important for embryogenesis in barley (*Hordeum vulgare* L.) (CHO, KASHA 1989).

Embryoids on regeneration media with high embryoid regenerating frequencies also had high green plant frequencies (Table 2). Each embryoid on average produced almost two plants. Embryoids on regeneration medium with 2.7 μM NAA, 2.3 μM kinetin and no $AgNO_3$ had the highest green plant frequency of 0.355. This result was similar to another study using embryoids of Pavon 76 on 190-2 regeneration medium with 2.7 μM NAA and 2.3 μM kinetin, or 24.6 μM indole-3-butyric acid and 0.3 μM gibberellic acid (0.318 and 0.236, respectively; ZHOU, KONZAK 1989). Plants from the same polyembryoid probably are clones (genetically identical), but also could be from separate microspores which fused during development. Clonal plants are useful as the doubling process is not assured and only 10-15% of the plants spontaneously double (SCHAEFFER et al. 1984).

The number of days from the anther plating to embryoid transfer was noted for each embryoid. Embryoid age ranged from 27 to 193 days after anthers were plated. The mean age was calculated for embryoids that produced green

Table 3. The number and mean age of embryoids producing no green plants or green plants, one green plant or multiple green plants

Embryoid regeneration	No. of embryoids	Mean age (days)
No green plants	1329	66.1 a*
Green plants	129	49.9 b
One green plant	77	50.9 a
Multiple green plants	52	48.3 b

* Entries followed by the same letter within the same group are not significantly different ($P < 0.05$) as determined by Chi-square statistical tests.

plants and for those embryoids which did not produce green plants. Regenerating embryoids (49.9 days) were observed to be significantly younger than non-regenerating embryoids (66.1 days) (Table 3). Ninety percent of the regenerating embryoids were transferred between 27 and 66 days after the anther plating. This result confirms a previous report, that early embryoids are more likely to regenerate plants than later embryoids (de BUYSER, HENRY 1979). Also, the younger embryoids were more likely to produce multiple plants (Table 3).

Table 4. The embryoid regenerating frequency and green plant frequency on each regeneration media

Basal salts	Plant growth regulators (μM)	Supplements or changes (μM)	Number of embryoids	Embryoid regenerating frequency	Green plant frequency
MS	2.7 NAA, 2.3 kinetin		225	0.15	0.40
MS	2.7 NAA, 2.3 kinetin	Amino acids[a]	54	0.28 +[b]	0.41
MS	2.7 NAA, 2.3 kinetin	130 mM cefotaxime	57	0.09	0.30
MS	2.7 NAA, 2.3 kinetin	2% PEG	63	0.02 –	0.03 –
MS	2.7 NAA, 2.3 kinetin	10 mM glucose	66	0.14	0.61 +
MS	2.7 NAA, 2.3 kinetin	60 AgNO3	63	0.25	0.35
MS	2.7 NAA, 2.3 kinetin	300 AgNO3	51	0.14	0.51
MS	2.7 NAA, 2.3 kinetin	60 Ag2S2O3	63	0.19	0.22 –
MS	2.7 NAA, 2.3 kinetin	300 Ag2S2O3	61	0.10	0.13 –
MS	2.7 NAA, 2.3 kinetin	50 ASA	54	0.09	0.09 –
MS	2.7 NAA, 2.3 kinetin	150 mM sucrose	66	0.18	0.83 +
MS	2.7 NAA, 2.3 kinetin	200 mM sucrose	63	0.03 –	0.16 –
MS	No plant growth regulators		66	0.21	1.06 +
MS	1.1 2,4-D		66	0.18	0.62 +
MS	2.7 NAA, 2.2 BAP		66	0.13	0.62 +
MS	5.4 NAA, 0.5 2iP		60	0.08	0.35
MS	5.7 IAA, 4.6 kinetin		66	0.14	0.55
MS	11.4 IAA, 8.9 BAP		63	0.08	0.13 –
MS	24.6 IBA, 0.3 GA3		57	0.09	0.18 –
85D12	2.7 NAA, 2.3 kinetin		279	0.14	0.44
85D12	2.7 NAA, 2.3 kinetin	Filter sterilized	129	0.24 +	0.88 +
P1	2.7 NAA, 2.3 kinetin		99	0.14	0.66
P2	2.7 NAA, 2.3 kinetin		96	0.05 –	0.20 –
N6	2.7 NAA, 2.3 kinetin		105	0.13	0.53
MN6	2.7 NAA, 2.3 kinetin		105	0.17	0.55

[a] The amino acids are 400 μM alanine, 400 μM arginine, 400 μM aspartic acid, 400 μM lysine, 400 μM proline, 400 μM serine, 1.2 mM asparagine, and 1.0 mM glutamine.

[b] Frequencies followed by + or – are significantly higher or lower ($P < 0.05$), respectively, than the respective frequency of MS medium containing 2.7 μM NAA and 2.3 μM kinetin with Bacto-agar agent as determined by Chi-square analyses.

The second regeneration experiment compared several regeneration media to the best regeneration medium in the first regeneration experiment (hereafter referred to as the control medium) which contained MS basal salts, 2.7 μM NAA, 2.3 μM kinetin, 58 mM sucrose and 0.76% Bacto-agar for their ability to regenerate green plants (Table 4). Embryoids on this medium had similar embryoid regenerating and green plant frequencies in both experiment 1 and 2 (Tables 2 and 4). Of the media tested in the second experiment, only two had significantly higher embryoid regenerating frequencies (Table 4). One medium was the control medium supplemented with amino acids. The second medium differed from the control in its salts (85D12) and was filter sterilized. Ethylene inhibitors, cefotaxime, many growth regulator concentrations, and basal media were not beneficial. P2 medium, increased osmoticums (2% PEG) or high levels of sucrose (200 mM) were detrimental.

While few media were superior to the control for embryoid regenerating frequency, several regeneration media had higher green plant frequencies than the control (Table 4). Beneficial changes included the addition of 10 mM glucose; increasing sucrose concentration to 150 mM; eliminating plant growth regulators; including only 1.1 μM 2,4-D; including 2.7 μM NAA and 2.2 μM BAP; filter sterilizing 85D12 medium constituents, and using P1 medium (Table 4). As expected, those media that were detrimental for embryoid regenerating frequency were also detrimental for green plant frequency.

The above study compares various media components for wheat anther culture in order to determine procedures that will efficiently produce large numbers of haploid regenerants. The need for a pre-culture cold treatment remains controversial. In light of previous work and this study, we recommend determining the genotypic need for cold pre-culture treatments. P1, 85D12, and N6, as constituted in this study, were similar for embryoid initiation. It is expected that the chemically defined media (85D12 and N6) will be more consistent than P1 which may vary with potato source. For plant regeneration, saccharide type and concentration, the presence or absence of plant growth regulators, and filter sterilization should continue to be studied for future improvements. These improvements, particularly the saccharide type and concentration, should be studied in media using different gelling agents (SIMONSON, BAENZIGER 1992) and over a wider range of cultivars. Ethylene inhibitors are not recommended as they were generally not beneficial except in low responding media which would not be used in doubled haploid breeding.

Acknowledgements. This research was supported by USDA grant No. 88000279, also published as paper No. 9231, Journal Series, Nebraska Agric. Res. Div.

Notes. This work was performed in connection with the FAO/IAEA Coordinated Research Programme on "The Use of Induced Mutations in Connection with Haploids and Heterosis in Cereals". Scientific editing of the paper was undertaken by Perry GUSTAFSON (Columbia, Missouri, USA) and Mirosław MALUSZYNSKI (FAO/IAEA, Vienna, Austria).

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