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Influence of media and growth regulators on somatic embryogenesis and plant regeneration for production of primary triticales

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Abstract

Basal media and plant growth regulators were tested for the promotion of somatic embryogenesis from immature wheat-rye hybrid embryos. Influence of growth regulators and chilling on plant regeneration were tested on two media. A medium containing four amino acids - glutamine, arginine, glycine and aspartic acid - as the nitrogen source, promoted the production of, on average, twice as much embryogenic callus as the other media, and somatic embryos developed well. The growth regulator dicamba was significantly better than 2,4-dichlorophenoxyacetic acid in promoting somatic embryogenesis and subsequent plant regeneration. Germination of somatic embryos on both regeneration media was enhanced by cold treatment. Supplementing 190-2 plant regeneration medium with a combination of α-naphthaleneacetic acid + benzyladenine, indole-3-acetic acid + kinetin or indole-3-acetic acid + zeatin resulted in equally high germination rates.

Abbreviations: 190-2 - Plant regeneration medium of Chuang & Jia; 2,4-D, - 2,4D Dichlorophenoxyacetic acid; Dicamba - 3,6-Dichloro-o-anisic acid; AA - Amino acid medium of Müller & Grafe; IAA - Indole-3-acetic acid; BA-Benzyladenine; NAA - α-Naphthaleneacetic acid

Introduction

Expansion of the genetic base of triticale (× *Triticosecale* Wittmack), a synthetic crop, is of major interest to plant breeders. Production of new amphiploids by crossing wheat (tetraploid wheat *Triticum durum* Desf., hexaploid wheat *Triticum aestivum* L.) and rye (*Secale cereale* L.), and subsequently doubling the chromosome number of the F1 hybrids, is used to exploit the rye and wheat gene pools (Varughese *et al.*, 1987). The major problem with tetraploid wheat×rye crosses is poor endosperm development and embryo abortion. Hybrid embryos from hexaploid wheat×rye crosses are more viable, but are usually few due to poor seed set. Embryo rescue and *in vitro* culture are useful in both cases (Kaltsikes & Gustafson, 1986).

Embryo culture is typically used for producing amphiploid plants (Kaltsikes & Gustafson, 1986). During the required chromosome doubling with colchicine, on average 70% of the hybrid plants are lost (Immonen *et al.*, 1993). Through production of embryogenic callus, many more plants can be obtained from a single embryo (Immonen, 1993b). Induction of embryogenic callus is important as most of the hybrid embryos are either small or abnormal, and unable to differentiate directly into plants (Shao & Taira, 1990). Attempts to regenerate plants can be repeated through callus subcultures. A broadly applicable callus culture protocol is needed, as the intergeneric F1 hybrid embryos are new genotypes with, in most cases, unknown *in vitro* response.

Somatic embryogenesis for cereals has been reported by many authors (Vasil, 1987; Bhaskaran & Smith,

1990). There are reports of the positive influence of amino acids on cell culture of Poaceae (Skokut *et al.*, 1985; Jenes & Pauk, 1989; Muyuan *et al.*, 1990; Shetty & Asano, 1991; Padgett & Leonard, 1994). Immonen (1993a) reported the usefulness of a medium containing solely organic nitrogen, in the form of amino acids - glutamine, arginine, glycine and aspartic acid - (AA, Müller & Grafe according to Thompson *et al.*, 1986, see Table 1) on production of embryogenic callus from immature triticale embryo explants. Several reports have suggested that dicamba is equally, or more suitable, than the frequently used 2,4-D, for promoting production of embryogenic callus with wheat and rye (Husinger & Schauz, 1987; Papenfuss & Carman, 1987; Carman *et al.*, 1988b; Zimny & Lörz, 1989).

In this study, AA medium was tested along with three basal media commonly used in cereal tissue culture, and the effect of dicamba was compared with that of 2,4-D for inclusion in a protocol for production of wheat x rye hybrid plants through somatic embryogenesis. The influence of growth regulators and chilling on germination of somatic embryos was studied on two basal media.

Materials and methods

Plant material

The parents were grown and the crosses made in the field at two locations: during the summer cycle at El Batan in the central Mexican highlands (19°31′ N, 98°50′ W, 2249 meters above sea level) under rainfed conditions with supplementary irrigation, and during the winter cycle at Cd. Obregon in the coastal region of northwest Mexico (27°20′ N, 109°54′ W, 39 meters above sea level) under irrigation. The following crosses were made: Yavaros 79×China/Snoopy, Sham 3×Selcurt/Snoopy and Tcho//Shwa/Mal/2/Crex×Turkey/Sardev (tetraploid wheat×rye crosses) for testing callus induction media (Test 1, summer cycle); Yavaros 79×China/Snoopy and Sham 3×Selcurt/Snoopy (tetraploid wheat×rye crosses) for testing growth regulators (summer cycle); SBA mutant×Sardev, Sham 3×Sardev (tetraploid wheat×rye crosses) and Lfn/1158.57//Prl/3/Hahn×Sardev (hexaploid wheat×rye cross) for testing callus induction media (Test 2, winter cycle).

The florets were pollinated twice; one day and four days after emasculation. The spikes were thoroughly sprayed with gibberellic acid (75 ppm) within an hour of pollination (Mujeeb-Kazi & Kimber, 1985). Immature caryopses were collected 16-17 days after second pollination. They were surface sterilized in 70% ethanol for 1 min, followed by 10 min in 1.2% sodium hypochlorite solution with 0.5% polyoxyethylenesorbitan monooleate (Tween 80), and thoroughly rinsed in sterilized water. For callus induction, the embryos were placed in sterile plastic petri dishes (35 mm diameter) containing 4 ml of medium, four per dish, scutellum side up and the embryo axis embedded in the medium.

Media and growth regulators

In Test 1, hybrid embryos were cultured on the following 5 media: AA, MS (Murashige & Skoog, 1962), 1/2MS (MS with half strength macro elements [He *et al.*, 1988]), N6 (Chu *et al.*, 1975) and modified Kao medium (Kao, 1977; Nakamura & Keller, 1982) without vitamins A, D_3 and B_{12} and coconut milk. The AA, MS, 1/2MS and N6 media were supplemented with 2% sucrose and Kao medium with 3% glucose and 1% sucrose. Each medium was supplemented with 9 μM 2,4-D. During the same crossing cycle dicamba and 2,4-D were tested at two concentrations, 9 and 18.1 μM, in MS medium for promoting somatic embryogenesis. During the following winter cycle, hybrid embryos were cultured on 4 media (Test 2): AA, MS, 2MS (MS with double strength inorganic salts, [Ozias-Akins & Vasil, 1983]) and N6, supplemented with 2% sucrose and, based on the results the previous cycle, with 18.1 μM dicamba.

Callus cultures were incubated at 24 °C in darkness for five weeks, after which embryogenic calli were transferred to 1/2 MS medium lacking growth regulators, for plant regeneration. These cultures were incubated at 24 °C in a 16-h photoperiod (50 μE m^{-2} s^{-1}, fluorescent light).

To study factors influencing plant regeneration, the effects of two basal media, 1/2 MS and 109-2 (Chuang & Jia, 1980, according to Wang & Hu, 1984; see Table 1), supplemented with 2% sucrose, were compared using embryogenic callus from hybrid embryos of one hexaploid wheat×rye cross (Lfn/1158.57//Prl/3/Hahn×Sardev) induced on AA medium with 18.1 μM dicamba. The following treatments were used: culture at 24 °C without plant growth regulators (TR1), culture at 8 °C for 6 days (TR2) or for 12 days (TR3), otherwise as TR1, culture at 24 °C with 5.71 μM IAA + 4.56 μM zeatin (TR4),

Table 1. Composition of AA callus induction medium and 190-2 plant regeneration medium (mg l^{-1}).

	AA	190-2
KNO_3		1000
KCl	2940	40
KH_2PO_4	170	300
$(NH_4)_2 \cdot SO_4$		200
$CaCl_2 \cdot 2H_2O$	440	
$MgSO_4 \cdot 7H_2O$	370	200
$Ca(NO_3)_2 \cdot 4H_2O$		100
$MnSO_4 \cdot 4H_2O$	22.3	8.0
$ZnSO_4 \cdot 7H_2O$	8.6	3.0
H_3BO_3	6.2	3.0
KI	0.83	0.5
$Na_2MoO_4 \cdot 2H_2O$	0.25	
$CuSO_4 \cdot 5H_2O$	0.025	
$CoCl_2 \cdot 6H_2O$	0.025	
$FeSO_4 \cdot 7H_2O$	27.85	27.85
Na_2EDTA	37.25	37.25
Myo Inositol	100	100
Nicotinic acid	0.5	0.5
Thiamine-HCl	0.5	1
Pyridoxine-HCl	0.1	0.5
Glycine	75	2
L-Glutamine	877	
L-Aspartic acid	266	
L-Arginine	228	

5.71 μM IAA + 4.44 μM BA (TR5), 2.85 μM IAA + 4.6 μM kinetin (TR6), 0.27 μM NAA + 2.22 μM BA (TR7) or 2.69 μM NAA + 4.65 μM kinetin (TR8). Sixty pieces of embryogenic callus, containing approximately 5 somatic embryos each, were used for each medium/treatment combination. The total amount of embryogenic callus and numbers of somatic embryos per medium and treatment were similar.

All media were agar-solidified (0.6% agar) and adjusted to pH 5.8 before sterilization. All components were autoclaved (15 min 121 °C 20 psi) with the exception of the vitamins, organic acids, sugars (except glucose and sucrose), dicamba, IAA and zeatin, which were filter sterilized and added to the sterilized media. In Test 1, embryos were also cultured on completely autoclave-sterilised AA medium.

Table 2. Frequencies of explant embryos (%) showing callus induction (CI), embryogenic callus (EC) and plant regeneration (R), and mean number of plants (PL) per explant embryo in two tests.

Media	N	CI	EC	R	PL
Test 1					
AA	71	63.4a	32.3a+	29.2a+	1.78a
N6	69	53.6ab	19.4b+	14.1b+	1.25a
KAO	71	42.3b	18.8b+	17.6ab+	1.49a
MS	69	42.0b	18.2b+	15.6b+	1.33a
1/2 MS	72	40.3b	17.6c+	16.4b+	0.58a
Test 2					
AA	100	75.0a	33.0a	28.0a	5.18a
N6	96	74.0a	7.9c	5.2c	0.78c
2 MS	99	64.6ab	20.2b	20.2ab	3.98ab
MS	94	57.4b	20.2b	14.9b	3.47abc

N= total number of plated embryos. Numbers in columns within a test with the same letter are not significantly different (CI, EC, R: χ^2, $p>0.05$, in columns marked with +, $p>0.01$; PL: LSD, $p>0.05$, calculated from data transformed by $\sqrt{(x+1)}$)

Observations and statistical analysis

The frequency of embryos forming callus and calli with embryogenic sections was noted after five weeks of culture. Embryogenic callus was smooth, nodular and compact, pale yellow in colour and formed somatic embryos. To assess the development rate and intensity of embryogenesis, each embryo was visually scored weekly (total of four times) using a scale of 0–10 (0 corresponding to no embryogenic callus, 1–10 to embryogenic callus and presence of somatic embryos). General scoring was opted for, as counting of individual somatic embryos was considered to be impractical and unreliable owing to large amounts of nodular embryogenic callus associated with pro-embryos and fused embryos. Frequency of calli forming complete plants was calculated after six and eight weeks of subculture. The number of plants was recorded.

Chi-square analyses (INSTAT, 1990) were used on frequency data. In the analysis of total plant production, embryos which did not produce plants were assigned a value of zero. The data on plant production were transformed to stabilise the variance effectively (Snedecor & Cochran, 1967) and analysed using a generalised linear model analysis of variance (SAS, 1985). Least significant difference was used to separate significantly different means. Mean scores for

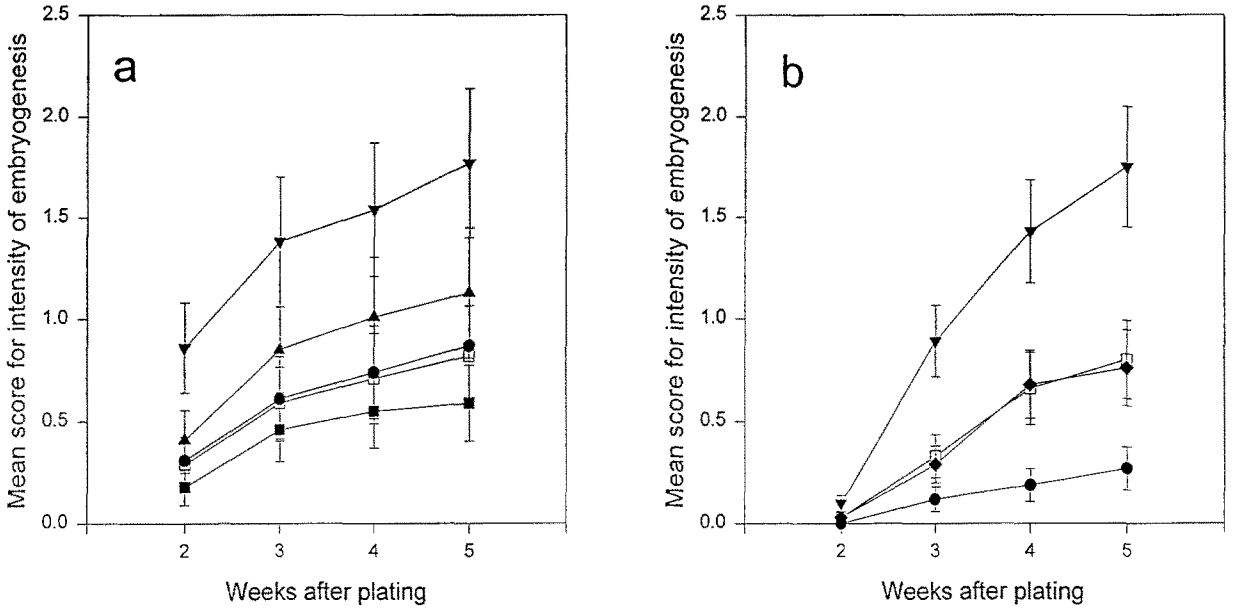

Fig. 1. Development of embryogenic calli from immature embryos during five weeks of culture; mean score (0–10) for intensity of embryogenesis (±SE); (*a*) Test 1. □, MS; ■, 1/2MS; ● N6; ▲, KAO; ▼, AA; (*b*) Test 2. □, MS; ♦, 2MS; ●, N6; ▼, AA.

Table 3. Frequencies of explant embryos (%) showing callus induction (CI), embryogenic callus (EC) and plant regeneration (R), and mean number of plants (PL) per explant embryo in test comparing growth regulators.

Growth regulator	Concentration μM	N	CI	EC	R	PL
2,4-D	9	48	41.7c	13.3c	7.1c	0.14c
2,4-D	18.1	47	53.2bc	27.3bc	15.8bc	0.12bc
dicamba	9	48	62.5ab	43.2ab	34.2ab	2.08ab
dicamba	18.1	46	76.1a	53.5a	43.2a	3.43a

N = total number of plated embryos. Numbers in the columns with the same letter are not significantly different (CI, EC, R:χ^2, $p>0.05$; PL: LSD, $p>0.05$, calculated from data transformed by $\sqrt{(x+1)}$)

the development rate and intensity of embryogenesis are presented graphically and include standard error bars.

Results

Callus induction

AA medium was significantly ($p < 0.05$) more efficient in promoting callus induction in Test 1 than the other media, with the exception of N6 (Table 2). In Test 2, AA and N6 media also promoted the most callus induction. Frequency of plated embryos forming embryogenic callus was highest on AA medium (Test 1: $p<0.01$, Test 2: $p<0.05$; Table 2).

Precocious germination of the embryo explant was only observed in Test 2 with embryos from the hexaploid wheat×rye cross (80.9% of embryos), on all media. However, on AA medium nearly half of the germinating embryos also produced embryogenic callus (data not shown).

The rate of development of embryogenic callus on the basal media tested is illustrated in Fig. 1. In Test 1, proliferation of embryogenic callus began early on AA medium (twice as much embryogenic callus as on other media after two weeks of incubation). In Test 2 the development of embryogenic callus began later, after two weeks of incubation, on all media, but the rate of development on AA medium was relatively fast and after five weeks of culture the intensity of somatic embryogenesis on AA medium was at least twice that on the other media tested. Well developed somatic embryos were frequent only on AA medium (Fig 2). However, plant yield from calli grown on AA medium was not in proportion to the quantity of somatic embryos, and their germination was often slower than regeneration of embryogenic callus produced on the other callus induction media. Plants regenerated from embryogenic callus produced on AA medium were

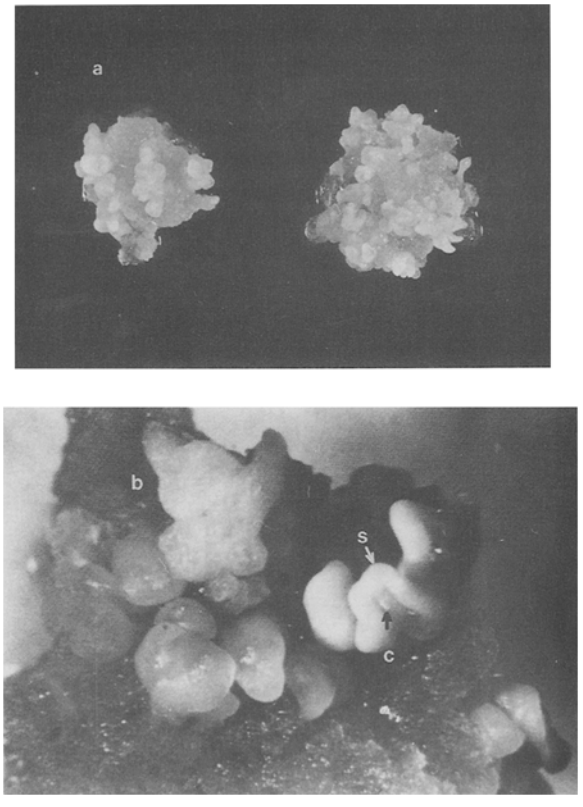

Fig. 2. Embryogenic calli on AA medium; (*a*) calli scored for intensity of embryogenesis as 5 (on the left) and 8 (on the right); (*b*) somatic embryos: s - scutellum, c - coleoptile.

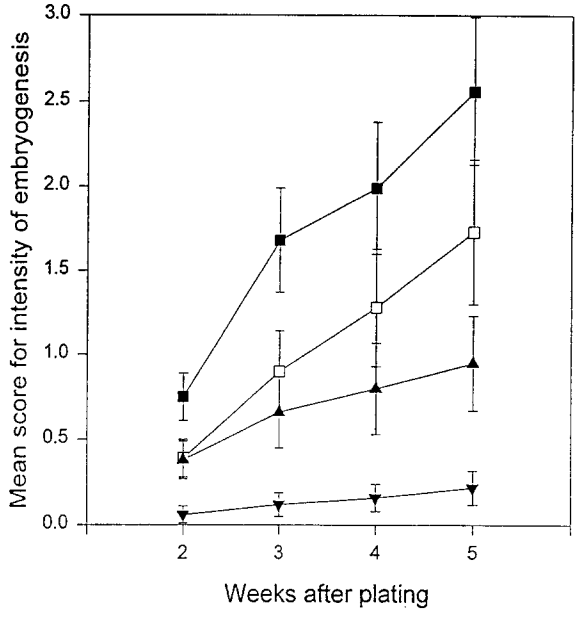

Fig. 3. Development of embryogenic calli from immature embryos during five weeks of culture on MS medium supplemented with 2,4-D or dicamba; mean score (0–10) for intensity of embryogenesis (± SE); ▼, 2,4-D 9 μM; ▲, 18.1 μM; □, dicamba 9 μM; ■, dicamba 18.1 μM.

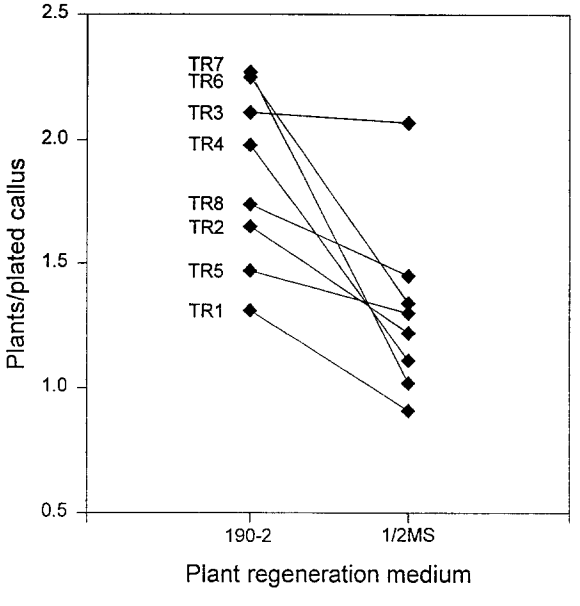

Fig. 4. Plant regeneration on 190-2 and 1/2MS plant regeneration media with the following treatments: TR1- culture at 24 °C without plant growth regulators; TR2 - culture at 8 °C for 6 days, otherwise as TR1; TR3 - culture at 8 °C for 12 days, otherwise as TR1; TR4 - TR8, culture at 24 °C with the following growth regulators: 5.71 μM IAA + 4.56 μM zeatin (TR4), 5.71 μM IAA + 4.44 μM BA (TR5), 2.85 μM IAA + 4.56 μM kinetin (TR6), 0.27 μM NAA + 2.22 μM BA (TR7), 2.69 μM NAA + 4.65 μM kinetin

generally more vigorous than those produced on other media.

Dicamba at 18.1 μM supported significantly more callus induction and proliferation of embryogenic callus per plated embryo ($p<0.05$) than either concentration of 2,4-D (Table 3). There was no significant difference between the effects of the two concentrations of dicamba when frequencies per plated embryo were considered (Table 3). The rate of development of embryogenic callus was much faster on media containing dicamba and on the medium with 18.1 μM dicamba, the intensity of embryogenesis reached a level nearly three times as high as that on media containing 2,4-D (Fig. 3). Plant regeneration from the embryogenic callus produced on media containing dicamba was higher than when 2,4-D was used (Table 3). No medium×cross interactions were detected for any of the tests.

Plant regeneration

In terms of total plant production, 190-2 medium was significantly more efficient than 1/2MS medium ($p<0.001$). Cold treatment for 12 days increased embryo germination significantly compared with all other treatments ($p<0.001$). On 1/2MS medium, 12 days of chilling (treatment 3) resulted in a total plant yield similar to that on 190-2 medium with treatments 7 (NAA + BA), 6 (IAA + kinetin), 3 (chilling for 12 days), and 4 (IAA + zeatin) (Fig. 4). There were no significant medium × treatment interactions for total plant production.

Discussion

In both tests, AA medium promoted formation of embryogenic callus and well-developed somatic embryos in more explants than the other media. This is important as many hybrid embryos are abnormal and very small when excised. With crosses which are difficult to make, even one successfully cultured embryo may give rise to several hybrid plants and the chances of getting an amphidiploid are therefore much improved.

In AA medium the nitrogen is solely in a reduced form and readily available for the plant cells. Padgett & Leonard (1994) showed that replacing all inorganic nitrogen with the same amino acids as in this study, clearly stimulated the growth of maize cells in suspension culture in comparison with media with nitrate nitrogen alone or together with ammonium nitrogen. They reported comparable results when glutamine or casein hydrolysate were added to a nitrate-based medium. Glutamine has been reported to be beneficial for cell cultures of several species (Henry & De Buyser, 1981; Olsen, 1987; Shetty & Asano, 1991). Olsen (1987) reported a significant increase in barley anthers responding with embryogenic structures by decreasing the concentration of ammonium nitrogen (from 20 mM to 2 mM) and adding glutamine (5.1 mM was optimal). In AA medium the concentration of glutamine is 5.96 mM. According to Gamborg (1970) glutamine could replace ammonium nitrate in soybean cultures. These results, which support previous findings by Immonen (1993a), suggest that inorganic nitrogen is not essential for induction of callus and embryogenesis with wheat×rye hybrids and triticale. Padgett & Leonard (1994) found glutamine to be particularly susceptible to autoclave sterilization, whereas the molecular stability of glycine, aspartic acid and arginine was not affected. With this in mind, the observation in this study, that there was no signifcant difference between autoclaved AA medium and partly filter-sterilized AA medium (data not shown) in promoting embryogenesis, indicates that glutamine alone may not account for the improved results. It cannot be argued on the basis of these results that all the four amino acids are essential and that a combination of ammonium or nitrate nitrogen together with amino acids would not further improve embryogenesis. The influence of the form of nitrogen (reduced/oxidised) or presence of amino acids may also depend on the auxin sensitivity of the plant cells. Grimes & Hodges (1990) suggested that glutamine may alter auxin sensitivity. In this respect 2,4-D and dicamba may elicit different responses. The results from the two tests on callus induction media are not comparable due to differences in plant materials, field conditions and growth regulators. The results on formation of embryogenic callus were nevertheless similar on AA and MS media in both tests, which may indicate lack of medium x auxin interaction in the case of these media. In contrast, the results with N6 medium were considerably poorer in Test 2 than in Test 1.

Total amount of nitrogen did not affect embryogenesis in this study, as the results for MS and 1/2 MS in Test 1 and MS and 2 MS in Test 2 did not differ from each other. The results on Kao medium, which has a similar nitrate:ammonium ratio as MS, but otherwise differs considerably from the other media tested, did not differ from those on MS.

AA medium also differs from the other media tested by containing a high level of KCl (39.4 mM), which according to Galiba & Yamada (1988) at a concentration of 40 mM, increased the formation of somatic embryos in wheat cultures.

Carman *et al.* (1988a) considered precocious germination of the embryo explant a useful parameter for assessing the effect of callus medium on somatic embryogenesis of *Triticum aestivum*. In this study precocious germination was cross specific and was almost solely observed with the hexaploid wheat×rye cross in Test 2. Thus, it did not appear to reflect the quality of the callus induction medium. However, in the experiment with plant growth regulators, dicamba suppressed all precocious germination, which confirms the findings of Papenfuss & Carman (1987).

In this study 2,4-D and dicamba were tested only at two concentrations, and the optimal growth regulator level, which may occur at an even higher concentration, has yet to be determined. With wheat, Carman

et al. (1988b) found no difference between concentrations of dicamba, ranging from 9.05 μM to 17.19 μM, in formation of somatic embryos up to four weeks in culture. The metabolism of dicamba is different to that of 2,4-D (Carman *et al.* 1988b) and the speed of metabolism depends on the plant species (Chang & Vanden Born, 1971).

Although the development of embryogenic callus and somatic embryos was highest on AA medium, the germination of the embryos was less than expected. This may have been due to increased dormancy during embryo development, as suggested by Carman (1988), or due to the residual effect of KCl in AA medium. Chilling for 12 days increased germination of somatic embryos, which confirms the observations of Rajasekaran *et al.* (1982) for *Vitis*. Longer periods could be tested, although chilling adds to the time required to produce amphihaploid plants. With the regeneration medium 190-2, similar results as for 12 days of chilling could be achieved using growth regulators. Experimentation on combined treatments of chilling and growth regulators might further improve germination of somatic embryos.

Callus culture was successful for all crosses used in the experiments. However, with crosses which showed poor response to tissue culture, it was not possible to draw conclusions on the usefulness of media and growth regulators, except that N6 medium completely failed to promote embryogenesis with the poorest crosses. Callus culture response of the hybrid embryos largely depends on the size and condition of the rescued embryo. AA medium and dicamba were efficient in triggering the *in vitro* development of hybrid embryos. Further development of a callus culture protocol based on the use of amino acids and dicamba may increase the chances for recovery of hybrid plants from wheat-rye combinations characterised by low crossability and from embryos of recalcitrant genotypes. Such a protocol might also be applicable for producing other intergeneric hybrids.

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