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Identification of callus types for long-term maintenance and regeneration from commercial cultivars of wheat (*Triticum aestivum* L.)*

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Summary. Immature embryos, inflorescences, and anthers of eight commercial cultivars of *Triticum aestivum* (wheat) formed embryogenic callus on a variety of media. Immature embryos (1.0–1.5 mm long) were found to be most suitable for embryogenic callus formation while anthers responded poorly; inflorescences gave intermediate values. Immature embryos of various cultivars showed significant differences in callus formation in response to 11 of the 12 media tested. No significant differences were observed when the embryos were cultred under similar conditions on MS medium with twice the concentration of inorganic salts, supplemented with 2,4-D, casein hydrolysate and glutamine. Furthermore, with inflorescences also no significant differences were observed. Explants on callus formation media formed two types of embryogenic calli: an off-white, compact, and nodular callus and a white compact callus. Upon successive subcultures (approximately 5 months), the nodular embryogenic callus became more prominent and was identified as 'aged callus'. The aged callus upon further subculture, formed an off-white, soft, and friable embryogenic callus. Both the aged and friable calli maintained their embryogenic capacity over many subculture passages (to date up to 19 months). All embryogenic calli (1 month old) from the different callus-forming media, irrespective of explant source, formed only green shoots on regeneration media that developed to maturity in the greenhouse. There were no significant differences in the response of calli derived from embryos and inflorescences cultured on the different initiation media. Also, the shoot-forming capacity of the cultivars was not significantly different. Anther-derived calli formed the least shoots. Aged and friable calli on regeneration media also formed green shoots but at lower frequencies. Plants from long-term culture have also been grown to maturity in soil.

Key words: Cereals – Gramineae – Somatic embryogenesis – *Triticum aestivum* – Wheat

Introduction

Plant regeneration from cultured cells and tissues is required for the successful application of biotechnology in crop improvement. Of importance too is the commercial value of the species and cultivars used in such research. Plant regeneration from tissue culture of many cultivars of wheat, one of the most important food crops in the world, has been well documented (Maddock 1985; Vasil and Vasil 1986). However, the highest frequencies of embryogenic callus and plant regeneration have been obtained from the culture of immature embryos (Ozias-Akins and Vasil 1982, 1983).

In this investigation we have assessed the capacity of immature embryos, inflorescences, and anthers to form embryogenic callus and plants on a range of media. Eight commercial wheat cultivars were examined to determine any possible relationship between cultivar and callus/plant formation. An important objective of this research was to recognize morphologically the different types of embryogenic calli formed in order to establish cell suspensions for use in protoplast culture and genetic transformation.

Materials and methods

Immature embryos, inflorescences, and anthers of eight commercial cultivars of wheat (*Triticum aestivum* L.) were used as

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Table 1. Cultivars and explant types used for callus formation and plant regeneration studies during 1987 and 1988

Cultivar	Explants		
	Immature embryos	Inflorescences	Anthers
Anza	1987, 1988		
Chris	1987, 1988		1988
Coker983	1988	1988	1988
FLA301	1987, 1988	1988	1988
FLA302	1987, 1988	1988	1988
Fremont	1987, 1988		
Hunter	1988	1988	1988
Pavon	1987, 1988		1988

Table 2. Embryogenic callus formation from immature embryos on different basal media. All field-grown material (1988 only)

Cultivar	% Embryogenic callus formation		
	MS	MSAA	C_8
Chris	76.1		15.8
Coker983	52.1	16.0	0.0
FLA301	76.0	23.7	5.0
FLA302	63.5	25.6	0.0
Pavon	61.3		17.5

explants for callus formation and later plant regeneration (Table 1). Explants were taken from plants grown in the field as well as in the greenhouse (immature embryos only). All field-grown material was provided by Dr. P. Bruckner (Coastal Plain Experimental Station, Tifton/GA). Field plantings were made between October and December in 1986 and 1987. For greenhouse-grown material, seeds were planted between January and March in pots containing Terra-Lite metro-mix growing medium. The growing season lasted approximately 3 months. In the first year of embryo culture only a few cultivars and media were tested. This preliminary work was repeated on a much larger scale in the second year and the results from both years were analyzed together.

Immature caryopses (10–12 days post-anthesis), and inflorescences (5–20 mm long, from young shoots prior to the emergence of the flag leaf) were surface-sterilized with 70% ethanol (30 s) and 20% NaOCl (10–15 min), followed by four changes of sterile distilled water. Immature embryos, 0.5–1.5 mm long, were dissected under a stereo dissecting microscope, and the inflorescences were cut into approximately 1-mm long segments, prior to culture. Spikes were pretreated at 4 °C in total darkness for 4–7 days before anther culture. Spikes were surface-sterilized with 70% ethanol (60 s), 10% NaOCl (10 min) and washed with four changes of sterile water. Anthers containing microspores at the uni-nucleate stage were aseptically removed and placed in culture.

Initially immature embryos were cultured on MS (Murashige and Skoog 1962), MSAA (Muller and Grafe 1978), and C_8 (Dudits et al. 1977) media with 3% sucrose and 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D; Table 2). Later, callus was initiated from inflorescences and immature embryos on MS medium with 2,4-D, dicamba, twice the concentration of MS inorganic salts (Ozias-Akins and Vasil 1983), potato extract (Chuang et al. 1978), casein hydrolysate, glutamine, silver nitrate, and cefotaxime. Cefotaxime was filter-sterilized and added to the autoclaved media (15 psi, 121 °C, 18 min) while all other additives were added to the media before autoclaving. Gelrite was added at 0.2%.

Anthers were cultured on solid and/or liquid N_6 (Chu et al. 1975), P_2 (Chuang et al. 1978), and WM2 (Datta and Wenzel 1987) media. Liquid cultures consisted of anthers dispersed in 2 ml medium in 5.5-cm petri dishes. The cultures were replenished with 1 ml fresh medium at 2-week intervals. Other media were solidified with 0.6% agar.

Large numbers of immature embryos, inflorescences, and anthers were cultured on each medium (Tables 3, 7, and 9). Immature embryos were cultured with the scutellum away from the surface of the growth medium. Embryo and inflorescence cultures were incubated at 28 °C in total darkness for 4 weeks. Anthers were incubated at 30 °C and total darkness for 6–8 weeks.

At the end of each growth period, embryogenic callus formation was assessed in all the explant types. Embryogenic calli were then transferred to regeneration as well as maintenance (inflorescences and embryos only) media. For regeneration, immature-embryo-derived embryogenic calli (1 month old) were transferred from the 12-callus formation media to MS medium with 1 mg l^{-1} indole-3-acetic acid (IAA) and 1 mg l^{-1} zeatin (Ozias-Akins and Vasil 1982). Later, aged and friable calli were placed on the same medium as well. Inflorescence-derived embryogenic calli (1 month old) were transferred to MS medium with 0.2 mg l^{-1} 2,4-D and 1 mg l^{-1} 6,$\gamma\gamma$-dimethylallylaminopurine (2iP) (Ozias-Akins and Vasil 1982) and calli derived from anthers on P_2 solid medium to MS medium with 0.5 mg l^{-1} naphthaleneacetic acid (NAA) and 0.5 mg l^{-1} kinetin (Ouyang et al. 1983). For each treatment, between 10 and 30 callus pieces were transferred to the regeneration media and maintained at 28 °C with a 16-h photoperiod, while anther-derived calli were incubated at 24 °C with a 16-h photoperiod. Shoot formation was assessed after 4 weeks.

For the maintenance of embryogenic callus, the more compact organized pieces, and later the friable calli as well, were transferred to maintenance medium and subcultured at 4-week intervals. Cultures were incubated at 28 °C in total darkness.

Regenerated plantlets, 10–15 mm high, were hardened by transfer to Magenta vessels containing MS medium without growth hormones and later to Conetainers with Terra-Lite metro-mix growing medium. After 2 weeks on a light cart, plants were transferred to the greenhouse. Plantlets derived from aged calli were also hardened by transfer to MS hormone-free medium or rooting media: MS (half strength) only, or MS (half strength) with varying concentrations of NAA, IAA, and indolebutyric acid (IBA). Plantlets were transferred directly from the regeneration or rooting media to pots of Vermiculite A3 for further rooting. Plantlets were maintained at 28 °C with a 16-h photoperiod and high humidity.

The data were analyzed assuming binomial sampling models using arc sin transformations on the proportion of callus and shoot formation. The weighted least-squares and the type 111 sum of squares analyses were used to draw conclusions on the basis of f-tests ($\alpha = 0.05$). The more interesting interactions were further analyzed by pair-wise multiple comparison tests at a 5% level of significance.

Results and discussion

Basal media

Immature embryos formed embryogenic callus on all three basal media tested (Table 2). However, callus for-

Table 3. Embryogenic callus formation from immature embryos on MS basal medium supplemented with different growth additives (1987 and 1988)

Cultivar	Year	Source tissue	Medium											
			% Embryogenic callus formation											
		Growth condition	MS1	MS2	MS2 P	MS(×2) 2	MS2 CH,G	MS(×2) 2,CH,G	MS2 Ag5	MS2 Ag10	MS2 Cf50	MS2 Cf100	MS 1D	MS 2D
Anza	88	Field	60.7	50.0	83.3	89.6	70.0	84.2	32.0	20.0	40.4	26.7	37.3	50.0
		G-H	53.3	41.4	50.0	56.7	70.0	54.2	35.0	16.7	23.3	6.7	33.3	44.8
	87	G-H		49.4				81.0						
Chris	88	Field	76.7	76.1	89.3	82.0	88.9	83.5	81.7	89.6	87.8	56.0	82.6	71.2
	87	G-H		45.0				73.2		56.3				
Coker983	88	Field	41.3	52.1	58.3	70.1	69.8	88.3	64.5	61.8	43.6	32.7	16.7	40.7
FLA301	88	Field	77.2	76.0	85.4	78.3	82.7	85.0	54.4	45.1	61.4	26.7	19.2	67.2
		G-H	70.0	83.9	72.7	95.0	82.9	91.2	55.0	50.0	80.0	66.7	25.0	90.5
	87	Field		48.8				69.3		76.0				
FLA302	88	Field	77.1	63.5	69.4	80.6	90.7	84.1	46.5	30.0	36.7	16.4	11.7	57.1
		G-H	60.0	100.0	70.0	86.7	75.0	90.0	33.3			30.0	20.0	80.0
	87	Field		53.3				70.5		57.8				
Fremont	88	Field	30.0	36.0	69.6	75.0	80.0	87.5	41.4	25.4	41.7	20.4	35.1	51.0
		G-H	47.5	50.0	72.2	46.0	64.7	71.8	46.6	33.3	36.8	44.9	16.0	19.0
	87	G-H		25.0				61.6						
Hunter	88	Field	46.7	55.0	63.3	63.3	53.3	70.0	61.7	60.0	28.3	32.8	12.0	43.3
		G-H	66.7	65.0	44.4	79.0	100.0	80.0	70.0	79.0	84.2	31.6	53.3	
Pavon	88	Field	61.3	61.3	57.1	69.6	60.0	77.0	48.6	56.3	74.0	36.7	35.1	82.0
		G-H	97.9	94.1	97.0	100.0	100.0	100.0	100.0	75.0	94.4	95.0	100.0	95.0
	87	G-H		57.5				46.4		20.0				
Min no.[a]		G-H	21	20	18	15	18	20	18	20	18	19	19	20
Max no.[b]		G-H	49	51	36	50	51	34	48	39	38	49	46	50
Min no.		Field	30	56	30	48	30	38	50	59	57	30	37	49
Max no.		Field	259	279	281	272	254	305	115	110	110	110	99	235

[a] Minimum number of embryos cultured
[b] Maximum number of embryos cultured
MS1 = MS + 1 mg l^{-1} 2,4-D; MS2 = MS + 2 mg l^{-1} 2,4-D; MS2P = MS + 2 mg l^{-1} 2,4-D + 5% potato extract; MS(×2), 2 = MS(twice concentration) + 2 mg l^{-1} 2,4-D; MS2CH,G = MS + 2 mg l^{-1} 2,4-D + 100 mg l^{-1} casein hydrolysate + 500 mg l^{-1} glutamine; MS(×2),2CH,G = MS (twice concentration) + 2 mg l^{-1} 2,4-D + 100 mg l^{-1} casein hydrolysate + 500 mg l^{-1} glutamine; MS2Ag5 = MS + 2 mg l^{-1} 2,4-D + 5 mg l^{-1} silver nitrate; MS2Ag10 = MS + 2 mg l^{-1} 2,4-D + 10 mg l^{-1} silver nitrate; MS2Cf50 = MS + 2 mg l^{-1} 2,4-D + 50 mg l^{-1} cefotaxime; MS2Cf100 = MS + 2 mg l^{-1} 2,4-D + 100 mg l^{-1} cefotaxime; MS1D = MS + 1 mg l^{-1} dicamba; MS2D = MS + 2 mg l^{-1} dicamba

mation on MS medium was significantly higher than on MSAA or C_8. Some cultivars formed no callus on C_8 medium. MSAA medium differs from MS medium in the replacement of ammonium and potassium nitrate with potassium chloride and an amino-acid mixture of glutamine, glycine, arginine, and aspartic acid. The replacement of MS nitrates with the amino-acid mixture was of no benefit to callus formation from immature embryos. The components of C_8 medium are different from those of MS or MSAA. In addition to the macro- and micro-elements, C_8 medium contains organic complexes such as urea, ammonium citrate, and yeast extract. Even though C_8 medium is specifically suited for the maintenance of cell suspensions initiated from mature-seed-derived callus of *Triticum monococcum* (Dudits et al. 1977), it supported very little growth of immature embryos of *T. aestivum*.

Media supplements for optimum response

Immature embryos of all cultivars formed embryogenic callus on the 12 media tested (Tables 3, 4, and 5). The percentage callus formation ranged from 6.7% to 100.0%. In many of the treatments, embryogenic callus formation was 50.0% or greater. There were significant differences between the 12 media tested (Table 4). Most

cultivars formed the highest percentage of embryogenic callus on the following 4 media: MS2P, MS(×2)2, MS2CHG, and MS(×2)2CHG (Table 3). Least embryogenic callus was formed on media with 1 mg l^{-1} dicamba and 100 mg l^{-1} cefotaxime. Cultivars responded similarly to MS medium supplemented only with 1 and 2 mg l^{-1} 2,4-D, 2 mg l^{-1} dicamba, as well as to 2 mg l^{-1} 2,4-D media augmented with 5 and 10 mg l^{-1} silver nitrate and 50 mg l^{-1} cefotaxime. Of the four media that gave the highest percentages of callus formation, most cultivars formed calli on media augmented with 100 mg l^{-1} casein hydrolysate, 500 mg l^{-1} glutamine, and with or without twice the concentration of MS inorganic salts. However, casein hydrolysate and glutamine replaced the requirement for twice the concentration of MS inorganic salts, since in the presence of these organic supplements the latter was not necessary. This effect was presumably due to the nitrogen supplement from glutamine. Consequently, MS medium containing 2 mg l^{-1} 2,4-D, 100 mg l^{-1} casein hydrolysate, and 500 mg l^{-1} glutamine was used as the maintenance medium for callus subcultures.

Cefotaxime at the concentrations used was without benefit when compared to media containing only 2,4-D (Tables 3 and 4). However, more embryos formed calli at the lower concentration of cefotaxime (50 mg l^{-1}) than at the higher level. Cefotaxime has previously been used in wheat to significantly improve growth, organogenesis, embryogenesis, and regeneration of immature-embryo-derived calli in culture (Mathias and Boyd 1986). It was reported that calli grew more rapidly on 60 and 100 mg l^{-1} cefotaxime than on media with 2,4-D alone. However, since no mention was made of the frequency of

Table 4. Data analysis for embryogenic callus formation from immature embryos in 12 media tested

Medium	LSMean	LSPhatm	
MS1[a]	0.9437	0.6557	C[b]
MS2	0.9115	0.6247	C D
MS2P	1.0365	0.7407	B
MS(×2)2	1.0761	0.7746	A B
MS2CH,G	1.1033	0.7969	A B
MS(×2)2CH,G	1.1449	0.8293	A
MS2Ag5	0.8801	0.5942	C D
MS2Ag10	0.8140	0.5285	D
MS2Cf50	0.8539	0.5683	C D
MS2Cf100	0.6614	0.3773	E
MS1D	0.6171	0.3349	E
MS2D	0.8797	0.5988	C D

[a] See Table 3 for abbreviations
[b] Treatments with the same letters are not significantly different

Table 6. Data analysis for embryogenic callus formation by eight cultivars in two growth conditions

Cultivar	LSMean Growth condition	
	Greenhouse	Field
Anza	0.38 Aa Ab	0.53 Ac B C
Chris		0.78 D
Coker983		0.52 C
FLA301	0.73 A B	0.67 A A
FLA302	0.64 A B C	0.60 A A B
Fremont	0.45 A A C	0.49 A C
Hunter	0.71 B B C	0.49 A C
Pavon	0.97 B D	0.59 A A B C

[a] Comparison between growth conditions
[b] Comparison within growth condition
[c] Treatments with the same letters are not significantly different

Table 5. Data analysis for response of cultivars to culture media. Field-grown material (1988 only)

Cultivar	Medium											
	MS1	MS2	MS2 P	MS(×2) 2	MS2 CH,G	MS(×2) 2,CH,G	MS2 Ag5	MS2 Ag10	MS2 Cf50	MS2 Cf100	MS 1D	MS 2D
Anza	B[a]	C	A	A	B	A	D	C	C	C	B	B
Chris	A	B	A	A	A	A	A	A	A	A	A	A
Coker983	C	C	C	B	C	A	B	B	C	B	C	C
FLA301	A	A	A	B	B	A	C	B	B	C	C	B
FLA302	A	A	B	A	A	A	C	C	C	C	C	B
Fremont	C	D	B	B	B	A	C	C	C	C	B	B
Hunter	BC	B	B	C	D	A	BC	B	D	B	C	C
Pavon	B	A	C	C	CD	A	C	B	B	B	B	A

[a] Cultivars with the same letters respond similarly to the culture media

Table 7. Embryogenic callus formation from inflorescences on MS basal medium supplemented with different additives. All field-grown material (1988 only)

Cultivar	% Embryogenic callus formation Medium											
	MS1[a]	MS2	MS2 P	MS(×2) 2	MS2 CH,G	MS(×2) 2,CH,G	MS2 Ag5	MS2 Ag10	MS2 Cf50	MS2 Cf100	MS 1D	MS 2D
Coker983	14.0	13.2	4.5	14.1	8.0	30.1	6.3	8.2	11.4	3.6	4.9	8.5
FLA301	32.3	27.3	17.0	38.1	7.0	12.0	6.7	8.3	12.0	13.3	5.7	5.6
FLA302	34.7	12.2	7.8	7.9	23.3	21.4	15.4	13.7	7.7	2.2	18.8	21.6
Hunter	20.2	12.4	8.8	20.0	18.8	19.7	11.1	9.5	4.4	3.4	7.1	7.0
Min no.[b]	93	82	51	63	32	59	91	85	90	93	85	88
Max no.[c]	124	121	130	120	115	113	120	124	108	116	106	141

[a] See Table 3 for abbreviations
[b] Minimum number of inflorescence segments cultured
[c] Maximum number of inflorescence segments cultured

Table 8. Data analysis for embryogenic callus formation from inflorescences in 12 media tested

Medium	LSMean	LSPhatm				
MS1[a]	0.5163	0.2437	A[b]			
MS2	0.4150	0.1626	A	B	C	
MS2P	0.3271	0.1032		B	C	D
MS(×2)2	0.4759	0.2099	A	B		
MS2CH,G	0.3478	0.1162		B	C	D
MS(×2)2CH,G	0.4733	0.2078	A	B		
MS2Ag5	0.3101	0.0931			C	D
MS2Ag10	0.3179	0.0977			C	D
MS2Cf50	0.3007	0.0877			C	D
MS2Cf100	0.2297	0.0518				D
MS1D	0.2892	0.0813			C	D
MS2D	0.3209	0.0995			C	D

[a] See Table 3 for abbreviations
[b] Treatments with the same letters are not significantly different

callus formation on the cefotaxime medium, the earlier results cannot be compared with the present studies.

In recent reports, dicamba has been tested for its effect on callus and embryoid formation in immature wheat embryos (Carman et al. 1987a, b). There was no difference between the two auxins with respect to callus formation, whether tested singly or in the presence of kinetin. However, dicamba significantly increased embryoid formation when compared to 2,4-D. In the present study, embryos responded similarly to dicamba and 2,4-D at 2 mg l^{-1} (Tables 3 and 4), but there was a tendency for immature-embryo-derived embryogenic callus to form embryoids more rapidly on dicamba media than on media with 2,4-D. More embryoids germinated on the lower than on the higher dicamba concentration.

Inflorescences of all cultivars formed embryogenic callus on the 12 media tested (Table 7). However, irrespective of the cultivars used, embryogenic callus formation occurred at much lower frequencies in inflorescences than in immature embryo explants. The percentage embryogenic callus formation ranged from 2.2% to 38.1%. As with the immature embryos, there were significant differences between the media tested (Table 8). Highest percentage of embryogenic callus formation occurred on MS media containing 1 mg l^{-1} 2,4-D as the only growth supplement, followed by media with twice the concentration of inorganic salts, both with and without the organic supplements. Cultivars responded similarly to all other growth media. Of the auxins, 2,4-D promoted more callussing than dicamba at both the concentrations used. The potato extract supplement, which proved beneficial for callus formation from immature embryos, was without benefit for inflorescence culture.

Cultivars and growth condition response

In 11 of the 12 media, significant differences were observed in the callusing ability of the eight cultivars tested for embryogenic callus formation using immature embryos (Table 5; $p=0.0000$). 'Chris' formed the highest percentage callusing and 'Coker983' formed the least callus. On medium containing twice the concentration of MS inorganic salts, with casein hydrolysate and glutamine all eight cultivars responded similarly. No significant differences were observed with the inflorescences (Table 7; p value $=0.1651$).

There was little significant difference in embryogenic capacity between field- and greenhouse-grown embryos (Table 6). Embryos from both growth conditions formed embryogenic calli at high frequencies (Table 3). Immature embryos of 'Anza', 'FLA301', 'FLA302', and 'Fremont' from the field and greenhouse responded similarly in culture (Table 6), while 'Hunter' and 'Pavon' immature embryos grown in the greenhouse formed more embryogenic callus than those of the field.

Table 9. Embryogenic callus formation from anthers on different media. All field-grown material (1988 only)

Cultivar	% Embryogenic callus formation Medium				
	N_6S	N_6L	P_2S	P_2L	WM2
Chris	1.8		7.0		
Coker983	1.0		2.2		
FLA301	0.0	0.0	0.2	0.2	0.0
FLA302	0.2	1.1	0.0	5.5	6.3
Hunter	0.0	0.2	7.2	0.5	0
Pavon	5.0		13.3		
Min no.	442	410	501	420	390
Max no.	1,139	470	1,081	830	620

S – solid; L – liquid

Explant and developmental stage

The developmental stage of the immature embryos was found to be the most important factor for the formation of embryogenic callus. Immature embryos, 1.0–1.5 mm long, showed the best response in culture irrespective of the media, cultivar, or growth condition of the source material. Embryos below or above this optimum size showed lower frequencies of embryogenic callus formation.

Of the three explants used, anthers responded most poorly in culture (Table 9). Embryogenic callus formation ranged from 0.0% to 13.3%. There were no marked differences between anthers cultured in solid or liquid media. Of the three media tested, most cultivars formed embryogenic callus on P_2 medium and least on WM2. 'Chris', 'Hunter', and 'Pavon' showed maximum response on the P_2 medium.

Callus type and long-term maintenance

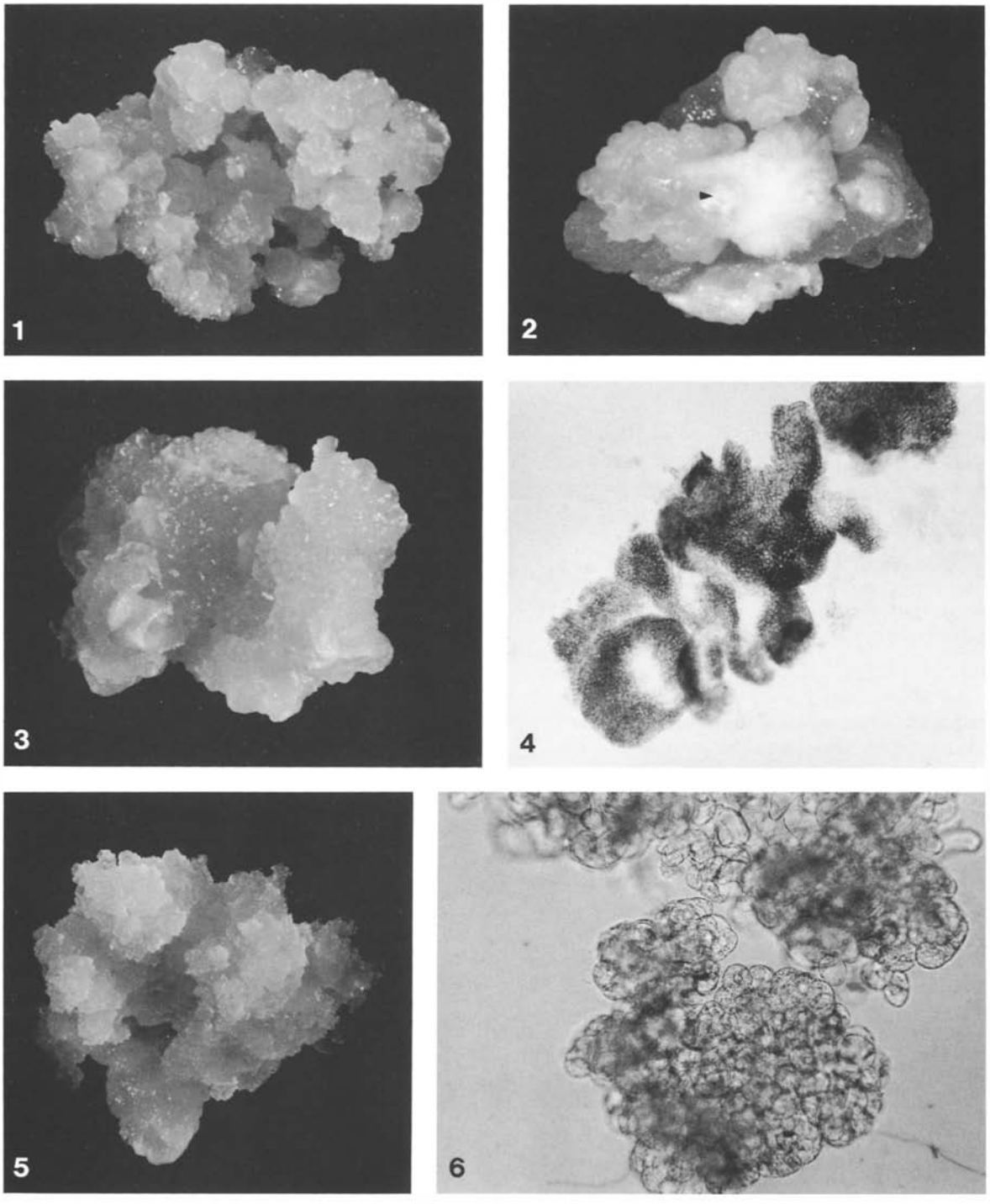
Explants cultured on the different callus formation media formed embryogenic as well as the soft, watery, nonembryogenic calli. Within a month of culture two types of embryogenic calli were observed. Immature embryos and inflorescences formed an off-white, compact, nodular, and organized callus (Fig. 1). In some instances, few of the explants formed another callus type, a white, compact embryogenic callus (Fig. 2; Ozias-Akins and Vasil 1982, 1983). Anthers in culture formed only the white compact type of embryogenic callus along with nonembryogenic callus. Although somatic embryos were more distinct on the white compact callus than on the off-white type, the white callus was difficult to subculture as it became brown and ceased to grow on maintenance medium.

The immature-embryo- and inflorescence-derived off-white, compact, and nodular embryogenic calli became less organized, less compact, and formed more soft nonembryogenic callus upon transfer to maintenance medium. Therefore, at each subculture, only the more compact and organized pieces were selected and transferred to fresh media. Inflorescence-derived calli after two subcultures became completely brown and necrotic and could not be maintained further. After five successive subcultures (approximately 5 months), the off-white, compact, nodular-organized, immature-embryo-derived embryogenic callus became more prominent in a few cultures (Fig. 3). Superficially this callus, referred to as 'aged callus', was similar to the previously formed 1-month-old callus (Fig. 1). Microscopically, the aged embryogenic callus was also similar to the 1-month-old callus; nodules with distinct epidermis were observed, with small, round, and densely cytoplasmic underlying cells (Fig. 4). An off-white, soft, and friable embryogenic callus was obtained after further subculture of the aged compact callus (Fig. 5). Microscopically, the friable callus consisted of groups of small, round, densely cytoplasmic cells interspersed with large, elongated, and highly vacuolated nonembryogenic cells (Fig. 6). The friable callus grew much faster than the compact callus. At each subculture the friable callus almost doubled in size and eventually consisted almost entirely of friable callus only. Compact calli grew slower and consisted of more nonembryogenic cells than the friable type.

During 1987 the aged and friable calli were only observed in one of the six cultivars tested, cv 'FLA302', occurring at a very low frequency (0.5%). However, during 1988 the two callus types were obtained in seven of the eight cultivars tested ('Chris', 'Coker983', 'FLA301', 'FLA302', 'Fremont', 'Hunter', and 'Pavon') at much higher frequencies (approximately 10%). The morphological distinction between the aged and friable calli was not always clear and in some cultivars a less compact, more friable callus first appeared within 2–5 months of subculture. Upon subculture, some callus pieces became even less compact, resulting in the formation of the friable callus. The aged as well as the friable calli of all the cultivars maintained their embryogenic capacity for many subculture passages (to date, up to 19 months).

Shoot formation

Immature-embryo-derived embryogenic callus (1 month old) readily formed green shoots on the regeneration medium. There were no significant differences (p value = 0.0588) in the response of calli derived from embryos cultured on the different initiation media. Cultivars formed shoots at high frequencies (up to 100% in most instances) from all the 12 callus initiation media tested, with no significant differences observed between them (p value = 0.8612). Initiation media containing silver nitrate or cefotaxime did not show any enhancement



Figs. 1–6. **1** Immature-embryo-derived, off-white, compact, nodular embryogenic callus formed within 1 month on callus formation media. **2** Immature-embryo-derived, white, compact embryogenic callus (*arrow*) formed within 1 month on callus formation media. **3** Off-white, compact, nodular, immature-embryo-derived embryogenic callus formed from aged cultures (5 months) on maintenance medium. **4** Microscopic view of aged off-white, compact embryogenic callus consisting of nodules with densely cytoplasmic cells. **5** Off-white, soft, friable embryogenic callus formed from aged cultures after 15 months on maintenance medium. **6** Microscopic view of friable callus consisting of groups of densely cytoplasmic cells interspersed with nonembryogenic cells

of shoot formation, as has been reported with other varieties (Purnhauser et al. 1987; Mathias and Boyd 1986).

The inflorescence-derived calli formed green shoots on all the 12 media tested, and no significant differences were observed between the media (p value = 0.0674). Also, the shoot-forming capacity of the cultivars was not significantly different (p value = 0.6159). However, when compared with immature-embryo-derived calli, the frequency of shoot formation was lower (50%–60%).

Of the three explant types, callus and shoot formation occurred at lowest frequencies from anthers and anther-derived calli, respectively (Table 9). On the regeneration media, green shoot formation ranged from 0.4%–18.1%.

Aged callus of cv 'FLA302' from 1987 readily formed green shoots at high frequencies (up to 70%) on the regeneration medium. The friable callus of this cultivar only formed embryoids on regeneration and maintenance media, as well as on MS medium with different concentrations of 2,4-D, 2iP, 6-benzyladenine, kinetin, gibberellic acid, and silver nitrate. One albino shoot was formed on the maintenance medium and transfer to regeneration medium did not improve shoot growth. In 1988 'Chris', 'Coker983', 'FLA301', 'FLA302', 'Fremont', 'Hunter', and 'Pavon' all formed green shoots on transfer of the less compact, more friable callus to regeneration medium.

Green shoots obtained from 1-month-old calli derived from immature embryos, inflorescences, and anthers formed roots on the regeneration medium. On transfer to MS hormone-free medium, plantlets increased in size and roots became more extensive. Shoots developed to maturity in the greenhouse.

Green shoots formed from aged immature-embryo-derived calli of all cultivars had poorly developed roots, and transfer to MS hormone-free medium or the different rooting media did not improve root growth. In Vermiculite shoots developed few roots. However, maintaining the plants in a humidity chamber improved growth, allowing transfer of plants to soil and growth to maturity in the greenhouse.

In conclusion, immature embryos, 1.0–1.5 mm long, were found to be the most suitable explants for embryogenic callus formation; anthers responded most poorly in culture. Using immature embryos, significant interactions were observed between the eight cultivars and the efficiency of callus formation on the media tested. However, no significant differences were observed in the callusing ability of the cultivars when inflorescences were cultured. Also, with immature embryos, all cultivars responded similarly to media containing twice the concentration of MS inorganic salts. These results tend to support the hypothesis that differences in cultivar response are physiological in nature and can be overcome by the culture condition and media (Vasil 1987). Recently, it has been shown that in maize "the induction of somatic embryogenesis in vitro is largely a physiological phenomenon. It is greatly influenced by the concentration and molecular structure of a given PGR and, under the appropriate conditions, can be achieved at relatively high frequencies independent of the genetic background of the explant tissue" (Close and Gallagher-Ludeman 1989). All embryogenic calli derived from immature embryos, inflorescences, and anthers formed green shoots on regeneration media. After successive subcultures on maintenance medium, immature-embryo-derived, off-white, compact, and nodular embryogenic callus formed aged as well as friable callus types, which maintained their embryogenic capacity over many subculture passages and formed green shoots on transfer to regeneration media. These callus types have been used to successfully establish embryogenic cell suspension cultures (Redway et al. 1990). Protoplasts isolated from such suspensions form green plants (Vasil et al. 1990).

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