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Plant regeneration in vitro from embryogenic cultures of spring- and winter-type barley (*Hordeum vulgare* L.) varieties

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Summary. Immature embryos of 41 lines of barley were screened in vitro for callus induction and somatic embryogenesis on different media to establish totipotent cultures. The use of modified MS and CC media, both supplemented with 1 g/l casein hydrolysate, and the substitution of agarose for agar resulted in the highest frequencies of somatic embryo induction. Embryogenic callus was induced and plants regenerated from 23 of the lines tested. The auxins 2,4-D, dicamba, picloram and 2,4,5-T were suitable for embryogenic callus induction. High frequencies of somatic embryo germination occurred on CC medium supplemented with 1 mg/l IAA and 0.05 mg/l zeatin. A strong genotypic effect on the capacity and frequency of embryogenic callus formation was found. Cultivar Golden Promise always gave the best results. Experiments with field grown material in 3 consecutive years showed that environmental factors also strongly influenced the induction of somatic embryogenesis and plant regeneration.

Key words: Barley (*Hordeum vulgare* L.) – Immature embryos – Somatic embryogenesis – Plant regeneration – Genotypes

Introduction

In vitro culture is an experimental tool in plant breeding only when efficient and reproducible plant regeneration systems are available. In barley different explant sources have been used for the induction of regenerable callus cultures: apical meristems (Cheng and Smith 1975; Weigel and Hughes 1985), immature embryos (Dale and Deambrogio 1979; Hanzel et al. 1985; Thomas and Scott 1985; Goldstein and Kronstad 1986), mature embryos (Lupotto 1984), immature ovary tissue (Orton 1979), immature inflorescences (Thomas and Scott 1985) and seedling mesocotyls (Jelaska et al. 1984; Rengel and Jelaska 1986). Plant regeneration from in vitro cultures is possible via somatic embryogenesis and organogenesis. Thomas and Scott (1985), Rengel and Jelaska (1986) and Weigel and Hughes (1985) regenerated barley plants from somatic embryos. The initiation of an embryogenic cell suspension has also been reported from which albino plants could be regenerated (Kott and Kasha 1984). In comparison with other cereals, however, reports of somatic embryogenesis in barley are rare (see Bright and Jones 1985). While Hanzel et al. (1985) examined effects of genotypes and medium composition on barley organogenesis, little information on the influence of these factors on somatic embryogenesis is available.

In this report we describe the induction in barley of embryogenic callus cultures and regeneration via somatic embryogenesis using immature embryos as explants. Our purpose was to examine genotypic influences and to determine effects of media composition on totipotent callus induction and plant regeneration.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; 2,4,5-T: 2,4,5-trichlorophenoxyacetic acid; dicamba: 3,6-dichloro-o-anisic acid; picloram: 4-amino-3,6,6-trichloropicolinic acid; NAA: naphtaleneacetic acid; IAA: indole-3-acetic acid; ABA: abscisic acid; BAP: 6-benzyl amino purine; 2iP: 6-(3-methyl-2 butenyl 1-amino)purine; GA_3: gibberellic acid

Materials and methods

Materials

In total 31 spring and 10 winter genotypes were field-grown during 1984, 1985 and 1986 for culture experiments. Greenhouse-grown plants of the cultivars Dissa and Golden Promise were

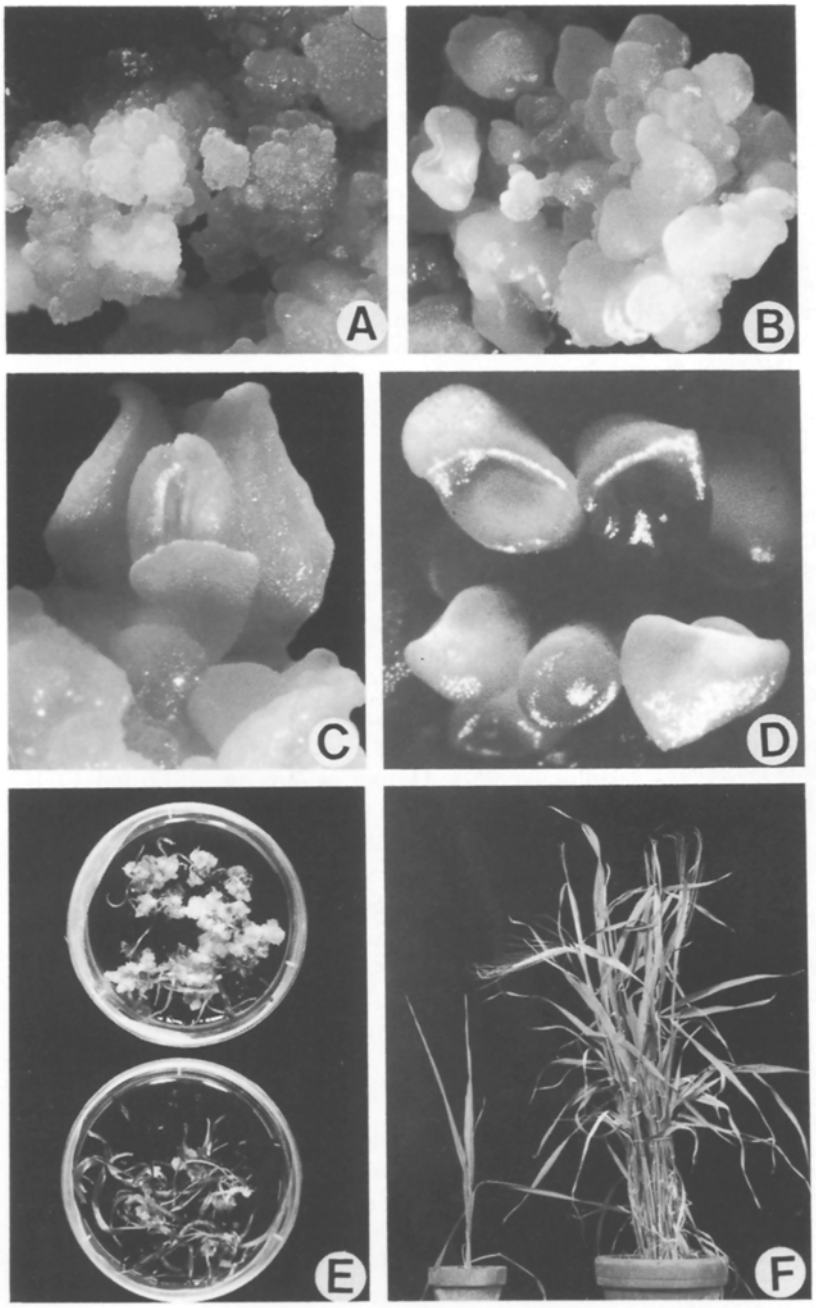

Fig. 1. **A** Friable callus developing from immature embryos of cv Himalaya 4 weeks after culture initiation on CC102 medium. **B** Embryogenic callus growing from explants of cv Golden Promise 2 weeks after culture initiation on CCOM2+C. **C** Single somatic embryo formed on 4-week-old callus culture derived from immature embryo of cv Golden Promise. **D** Embryogenic callus derived from immature embryo of cv Dissa 4 weeks after culture initiation. Somatic embryos of different developmental stages are visible on the callus surface. **E** Germination of somatic embryos. Somatic embryo germination begins on induction medium (*upper Petri dish*), but higher regeneration frequencies are obtained by transferring cultures to regeneration media (*lower Petri dish*). **F** Regenerants growing to maturity under greenhouse conditions (*left:* 6 weeks after transfer to soil; *right:* 12 weeks after transfer; both regenerants of cv Dissa)

also used for studying media and culture conditions. The barley lines were kindly provided by the Landessaatzuchtanstalt Stuttgart-Hohenheim and by Mr. M. Vater and Prof. J. Hesselbach of the Max-Planck-Institut in Köln-Vogelsang.

Explants

Dehusked immature seeds (10 to 14 days post anthesis) were treated with 70% ethanol for 1 min, with 1% NaOCl solution for 30 min and rinsed 3 times with sterile water. Immature embryos (0.8–2.0 mm in length) were then excised aseptically under a stereo microscope. In the experiments during 1984 whole embryos were cultured; in later experiments the coleorhizal and coleoptilar regions of the embryos were removed. Embryos longer than 1.5 mm were cut transversely in two or three pieces (Stolarz and Lörz 1986). Then 10 to 15 embryos were plated on 12 ml of medium in 60×15 mm Petri dishes (Greiner) with the embryo axis or cut surface in contact with the medium and cultured at 26 °C ± 1 °C in darkness.

Genotype screening

In 1984 immature embryos of 35 cultivars were cultured on four different media. Two basal MS (Murashige and Skoog 1962) media, both containing 2 mg/l 2,4-D, were used: one (MS12) supplemented with 30 g/l sucrose, the other (MS23) with 60 g/l sucrose and 100 ml/l coconut water (Gibco Lab). The third medium (B5-5) was basal B5 medium (Gamborg et

al. 1967) with 2 mg/l 2,4-D, 0.5 mg/l kinetin, 30 g/l sucrose, 100 mg/l glutamine and 160 mg/l proline and oxo-proline. Additionally, CC medium (Potrykus et al. 1979) was tested with supplements as published (2 mg/l 2,4-D, 20 g/l sucrose, 100 ml/l coconut water and 36.4 g/l mannitol) (CC102 medium). The media were prepared with 8 g/l agar (Merck) and autoclaved for 20 min at 121 °C and 1.0 kg/cm² after adjusting the pH to 5.8.

Media screening

Greenhouse material of the cvs Golden Promise and Dissa were used to examine different basal media and various additives. The following basal media were tested: MS (Murashige and Skoog 1962), CC (Potrykus et al. 1979), B5 (Gamborg et al. 1968), N6 (Chu et al. 1975), Norstog (1973), and the media of Kao (1977) with modifications described by Stolarz and Lörz (1986). Routinely, the media were supplemented with 2 mg/l 2,4-D and 30 g/l sucrose (N6 medium also with 4 or 8 mg/l 2,4-D). As MS and CC gave the best results, they were subsequently supplemented with different concentrations of following auxins: 2,4-D, dicamba, picloram, 2,4,5-T and NAA. 2,4-D, 2,4,5-T and NAA were tested in the range 1 to 8 mg/l, dicamba and picloram in the range 0.1–8 mg/l. Different concentrations of thiamine (0.5–5 mg/l), myo-inositol (0.25–1 g/l) and casein hydrolysate (Merck, acid hydrolysed and vitamin-free) (0.1–1 g/l) were added to MS and CC medium. Several amino acids, separately or in combinations (proline: 100 mM; alanine: 100 mM; arginine: 1 mM; tryptophane: 10 mM; cysteine: 10 mM) added to MS and CC medium were tested. All media were filter sterilized and later combined with the gelling agent, either agar or 8 g/l SeaPlaque agarose. The agar or agarose was autoclaved in deionised water.

Regeneration media and conditions

Embryogenic calli of cvs Golden Promise and Dissa were transferred for germination of somatic embryos to MS or CC media supplemented with different hormones and concentrations of hormones, sucrose and coconut water or to Norstog medium without any hormone. Cultures were maintained under low light intensity (250 lux) with a 16 h photoperiod at 24 °C ± 2 °C. Plantlets were transferred to half strength MS medium with 8 g/l Merck agar and 1% sucrose for root and shoot development and cultured under higher light conditions (Osram Natura, 2,000 lux, photoperiod 16 h, temperature 21 °C ± 2 °C). Then 3 to 4 weeks later regenerants were transplanted to pots (diameter: 6 cm) containing a soil-sand mixture (3 : 1) and grown under high humidity at 20 °C ± 4 °C with natural light for approximately 2 weeks. After acclimatization, plants were grown to maturity in the greenhouse with supplementary light (16 h) and a day/night temperature regime of 20 °C/12 °C. Regenerants of winter-type varieties were vernalized for 6 weeks at 6 °C.

Standard culture cycle

Cultures were scored for the formation of friable and embryogenic callus 4 to 6 weeks after plating. The responses of the cultured embryos were classified as follows:
(1) unresponsive or poorly-responsive embryos: no callus development or swollen, necrotic scutelli only; (2) soft, watery, translucent, non-morphogenic callus; friable, well-growing, white-yellowish callus (Fig. 1 A); (3) embryogenic, compact callus with smooth surface, usually in combination with friable callus (Fig. 1 B).

Immature embryos from field grown plants (1985 and 1986) were plated on four media: MS1 medium corresponding to basal MS medium supplemented with 2 mg/l 2,4-D, 30 g/l sucrose, 1 g/l casein hydrolysate and the standard MS vitamin concentration (0.4 mg/l thiamine, 100 mg/l inositol); MS50 medium equal to MS1, but with 1 mg/l thiamine and 250 mg/l inositol; CC102 + C medium, a CC medium containing 2 mg/l 2,4-D, 20 g/l sucrose, 1 g/l casein hydrolysate and mannitol; CCOM2 + C medium equal to CC102 + C but without mannitol. Then 3 to 6 weeks later embryogenic calli were transferred to CC medium supplemented with 1 mg/l IAA and 0.05 mg/l zeatin. The resulting plantlets were cultured as described above. Compact portions were again cultured on fresh medium to induce embryogenesis. The transfer of embryogenic calli to regeneration medium and of compact callus parts to induction medium continued for a few more cycles.

Results

Genotype screening

The aims of the first experiment were to identify suitable barley genotypes for establishing in vitro cultures and to examine the effects of media composition on callus induction. In this experiment 36 genotypes were tested on the media CC102, MS12, MS23 and B5-5 (Table 1). On MS23 and B5-5 media the scutelli of most genotypes became swollen and necrotic, with the exception of cv C143/82 which developed embryogenic callus on B5-5. During culture on MS12 and CC102 media, 21 of the lines tested produced fast-growing, friable calli (Fig. 1 A). Also, 15 of the 21 lines which produced friable calli developed an embryogenic type of callus (Fig. 1 B). Plants could be regenerated and transferred to soil from 8 of these 15 lines. Immature embryos of cv Golden Promise produced well-growing embryogenic callus. This cultivar, together with cv Dissa, showed the best in vitro regeneration capacity.

Screening for efficient induction and regeneration media

Due to the results obtained in the initial screening experiment, we used greenhouse-grown Golden Promise and Dissa for further experiments designed to improve induction media and regeneration conditions. Six basal media consisting of the basal salts of MS, CC, N6, B5, Kao and Norstog media, each with the addition of 2 mg/l 2,4-D and 30 g/l sucrose, were tested. This combination of sucrose and auxin had previously proved effective in combination with MS and CC medium. On Kao, Norstog and B5 media, slow callus growth and subsequent necrosis was observed. Culture on N6 medium led to a fast-growing, non-morphogenic callus. Also, higher 2,4-D concentrations did not induce morphogenesis with this medium. On MS and CC callus, growth was fast and embryogenic cultures were produced. Further experiments concentrated on these two media.

In MS and CC media, the auxins 2,4-D, 2,4,5-T, dicamba, picloram and NAA were tested. At the concen-

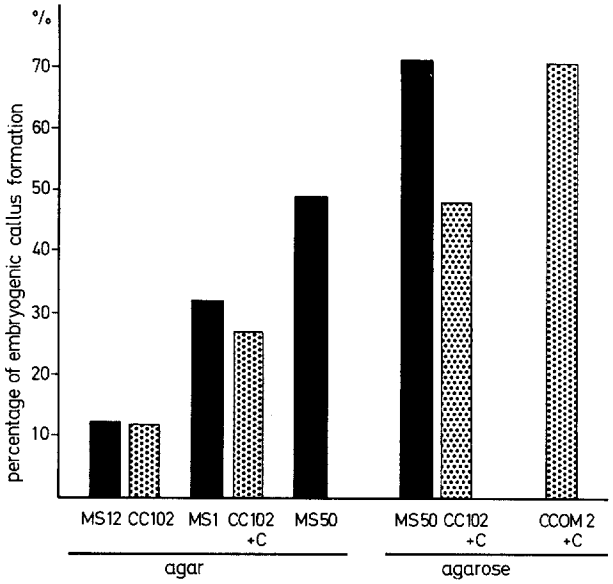

Fig. 2. Combined data for the frequency of embryogenic callus formation from explants of both cvs Golden Promise and Dissa (greenhouse material) on MS and CC media modified as follows: addition of 1 g/l casein hydrolysate (MS1, CC102+C); addition of 0.6 mg/l thiamine and 150 mg/l inositol to standard MS vitamins (MS50); replacement of agar (8 g/l) by 8 g/l SeaPlaque agarose (mannitol of the CC medium omitted; CCOM2+C)

Table 1. Responses of immature embryos of 31 spring-type and 5 winter-type barley lines

Line	No. of plated embryos	Percentage forming friable calli	Percentage forming embryogenic calli	Plant regeneration
Spring types:				
'Algerian'	140	20.0	0.0	–
'Aramir'	145	14.5	0.7	–
'Argentinische Futtergerste'	237	17.2	0.0	–
'Astrachan'	159	25.2	0.0	–
'Atem'	226	0.4	0.0	–
'Aura'	142	9.9	1.4	–
'Basar'	83	0.0	0.0	–
'Black Barley'	176	0.0	0.0	–
'Black Hull'	249	0.0	0.0	–
'Chiro Chinko'	182	0.0	0.0	–
'Dissa'	194	7.2	2.6	+
'Emir'	161	9.3	1.2	+
'Galore Can'	158	13.3	0.0	–
'Gemeiner Dreizack'	160	6.3	0.6	–
'Glabron'	185	0.0	0.0	–
'Golden Promise'	176	17.6	4.0	+
'Gopal'	159	2.5	0.0	–
'Himalaya'	145	2.8	2.1	+
'Imperial'	182	2.2	0.5	–
'Jane Hadaka'	186	0.0	0.0	–
'Local Sebergelon'	237	8.0	2.5	–
'Maris Mink'	249	2.8	0.8	+
'Midas'	168	2.4	3.0	–
'Oac Ottawa'	232	3.9	2.2	+
'Pfauengerste'	135	0.0	0.0	–
'Ricotense'	196	14.8	0.5	–
'SK 28/5'	151	0.0	0.0	–
'Violette'	196	0.0	0.0	–
'Wisconsin'	123	1.6	0.8	+
'WG R423'	92	0.0	0.0	–
'2-zeilige'	184	3.7	0.0	–
Winter types:				
'C 143/82'	106	0.0	5.7	+
'1585/82'	75	0.0	0.0	–
'1586/82'	27	0.0	0.0	–
'C 12/82'	23	0.0	0.0	–
'Vogelsanger Früh'	36	0.0	0.0	–

Table 2. Media compositions tested for the improvement of germination of somatic embryos. Basal media were MS or CC salts with supplements as indicated. st denotes coconut water and sucrose at standard concentration (CC: 20 g/l sucrose, 100 ml/l coconut water; MS: 30 g/l sucrose). The development of somatic embryos was scored as "–" no germination, "+" rare germination and "+ +" highly efficient germination of somatic embryos into plantlets

2,4-D (mg/l)	NAA (mg/l)	IAA (mg/l)	ABA (mg/l)	GA_3 (mg/l)	Kinetin (mg/l)	BAP (mg/l)	2iP (mg/l)	Zeatin (mg/l)	Zeatin riboside (mg/l)	Coconut water (ml/l)	Sucrose (g/l)	Germination of somatic embryos
–	–	–	–	–	–	–	–	–	–	st	20	+
–	–	–	–	–	–	–	–	–	–	st	30	+
0.1	–	–	–	–	–	–	–	–	–	st	st	–
0.5	–	–	–	–	–	–	–	–	–	st	st	–
–	1.0	–	–	–	–	–	–	–	–	st	st	–
–	–	1.0	–	–	–	–	–	–	–	st	st	–
–	–	–	0.01	–	–	–	–	–	–	st	st	+
–	–	–	0.05	–	–	–	–	–	–	st	st	+
–	–	–	–	0.5	–	–	–	–	–	st	st	–
–	–	–	–	1.0	–	–	–	–	–	st	st	–
–	–	–	–	–	0.1	–	–	–	–	st	st	–
–	–	–	–	–	0.5	–	–	–	–	st	st	–
–	–	–	–	–	1.0	–	–	–	–	st	st	–
–	–	–	–	–	–	0.5	–	–	–	st	st	–
–	–	–	–	–	–	1.0	–	–	–	st	st	–
–	–	1.0	–	–	–	–	0.05	–	–	st	st	–
–	–	1.0	–	–	–	–	0.5	–	–	st	st	–
–	–	1.0	–	–	–	–	–	0.05	–	st	st	+ +
–	–	0.5	–	–	–	–	–	0.1	–	st	st	+
–	–	0.25	–	–	–	–	–	0.5	–	st	st	–
–	–	0.05	–	–	–	–	–	1.0	–	st	st	–
–	–	–	–	–	–	–	–	1.0	–	st	st	–
–	–	1.0	–	–	–	–	–	–	0.05	st	st	+ +
–	1.0	–	–	–	–	–	–	0.05	–	st	st	–
–	–	–	–	–	–	–	–	–	–	10	st	+
–	–	–	–	–	–	–	–	–	–	50	st	+
–	–	–	–	–	–	–	–	–	–	100	st	+

trations tested (2, 4 and 8 mg/l) NAA induced no embryogenic callus. However, MS and CC media supplemented with one of the other auxins induced an embryogenic callus. When concentrations of 2 and 3 mg/l 2,4-D were compared, few differences in culture response were observed, and higher levels of 2,4-D gave no visible increase in embryogenic callus induction. Media supplemented with dicamba showed highest induction frequencies at concentrations of 1 mg/l and 6 mg/l; picloram gave the best results at 2 mg/l, and 2,4,5-T at 1 mg/l (data not shown).

Several of the organic supplements had important effects on somatic embryogenesis. All concentrations and combinations of amino acids tested inhibited callus development. However, supplementing media with casein hydrolysate increased the induction of embryogenic callus. Also, levels of thiamine and myo-inositol higher than in MS led to a higher induction rate of embryogenic callus (Fig. 2). The major conclusions of the media screening experiments were that MS medium supplemented with 1 mg/l thiamine and 0.25 g/l myo-inositol (MS50) or CC medium without mannitol (CCOM2+C), both supplemented with 1 g/l casein hydrolysate and 2 mg/l 2,4-D and solidified with 8 g/l SeaPlaque agarose gave the highest frequencies of embryogenic callus induction (Fig. 2).

The next phase in the establishment of an efficient in vitro system for barley was to define a suitable regeneration medium. Following plating on primary induction media 3 to 4 weeks after culture initiation, somatic embryos in different developmental states could be observed on the surface of callus (Fig. 1C, D). Well-developed somatic embryos germinated in situ (Fig. 1E), while less differentiated structures re-callused. To increase the frequency of plantlet formation, somatic embryos were transferred to different regeneration media (Table 2). CC was the best basal medium and MS was also suitable, whereas Norstog medium, originally developed for the germination of immature sexual embryos in vitro, did not permit a further development of

Table 3. A 3 year comparison of the frequency of induction of friable and embryogenic callus from immature embryo explants of spring- and winter-type barley lines. Media used in 1985 and 1986 differed from those used in 1984; see "Material and methods"

Line	No. of explants			Percentage of explants forming friable calli			Percentage of explants forming embryogenic calli		
	1984	1985	1986	1984	1985	1986	1984	1985	1986
Spring types:									
'Aramir'	145	152	–[a]	14.5	43.4	–	0.7	19.1	–
'Aura'	142	140	–	9.9	56.4	–	1.4	31.4	–
'Dissa'	194	133	53	7.2	64.7	24.5	2.6	31.5	11.3
'Emir'	161	100	61	9.3	81.0	54.1	1.2	49.0	16.4
'Gemeiner D.'	160	144	–	6.3	39.6	–	0.6	19.4	–
'Golden Prom.'	176	140	84	17.6	80.0	51.2	4.0	63.6	28.8
'Himalaya'	145	234	–	2.8	84.6	–	2.1	20.1	–
'Jane Hadaka'	186	59	50	0.0	1.7	0.0	0.0	0.0	0.0
'Local Seberg.'	237	143	–	8.0	40.6	–	2.5	13.3	–
'Maris Mink'	249	164	–	2.8	44.5	–	0.8	29.9	–
'Midas'	168	143	59	2.4	60.8	42.4	3.0	42.0	11.9
'Oac Ottawa'	232	93	–	3.9	54.8	–	2.2	39.8	–
'Pfauengerste'	135	89	–	0.0	55.1	–	0.0	37.1	–
'Ricotense'	196	83	–	14.8	37.3	–	0.5	25.3	–
'Wisconsin'	123	92	–	1.6	59.8	–	0.8	45.7	–
Winter types:									
'Corona'	–	95	110	–	89.5	25.5	–	32.6	11.8
'Franka'	–	102	152	–	72.5	23.7	–	7.8	11.8
'Gerbel'	–	113	59	–	46.0	27.1	–	2.7	6.8
'Igri'	–	134	28	–	88.8	7.1	–	15.7	7.1
'Vogelsanger Früh'	36	112	80	0.0	85.7	1.3	0.0	4.5	1.3
'Vogelsanger Gold'	–	108	44	–	89.8	70.5	–	12.5	38.6
'C 143/82'	106	104	99	0.0	62.5	4.0	5.7	8.7	3.0
'1585/82'	75	–	40	0.0	–	10.0	0.0	–	7.5
'1586/82'	27	–	59	0.0	–	28.8	0.0	–	27.1

[a] Not tested

somatic embryos. Supplementing CC and MS media with different types and concentrations of hormones, or omitting hormones, led to the following observations. Decreasing the 2,4-D level or transferring embryos to hormone-free medium often resulted in recallusing of embryogenic structures. Short-term culture (1 week) on media containing ABA did not increase the germination rate, whereas certain cytokinins supported germination. Zeatin and zeatin riboside at concentrations of 0.05 mg/l applied in combination with IAA (1 mg/l) increased germination frequency, but BAP, kinetin and 2iP had deleterious effects, such as browning of the callus and necrosis of somatic embryos. Transfer of plantlets to half strength MS medium after 2 to 3 weeks on regeneration medium allowed vigorous root and shoot development which allowed planting in pots (Fig. 1 F).

Culture experiments with improved conditions

All lines which produced embryogenic calli in the first screening experiment of 1984 (except cv Imperial) were tested in 1985 and 1986 using the improved media. We also tested lines which were unresponsive or responded very poorly in the initial experiment, namely cvs Pfauengerste, Jane Hadaka and Vogelsanger Früh. Five new winter-type cultivars were added to this experiment.

The use of MS50, CC102+C and CCOM2+C for embryo culture clearly increased the frequencies of induction of friable and embryogenic callus (Table 3). For example, cv Pfauengerste, which did not produce any embryogenic callus in 1984, developed somatic embryos in 30% of cultures in 1985. In contrast, cv Jane Hadaka produced a watery type of non-morphogenic callus in all three seasons. Plants could be regenerated from all 24 genotypes tested under improved conditions, with the exception of cv Jane Hadaka. Out of 19 of these lines screened in 1984, only 14 of them developed embryogenic calli and eight regenerated plants (Table 1). Embryogenic callus formation and plant regeneration was also observed in each of the five winter-type lines tested only in 1985 and 1986. In 1985, winter-type lines showed lower frequencies of embryogenic callus formation than spring-type lines. Moreover, the formation of embryogenic callus in both spring- and winter-type cultivars was lower in 1986 than in 1985 (Table 3), with the exception of cv Vogelsanger Gold. In 1984 and 1985, the

Table 4. Plant regeneration from 14 spring-type barley lines. Immature embryo-derived cultures were transferred after 3 to 6 weeks to regeneration medium and the number of plants obtained (from the first subculture cycle only) recorded

Line	No. of calli transferred to regeneration medium	No. of calli giving rise to plants in soil (%)	No. of plants established in soil	No. of fertile plants (%)
'Aramir'	9	9 (100)	9	9 (100)
'Aura'	23	14 (60.8)	30	30 (100)
'Dissa'	28	28 (100)	42	42 (100)
'Emir'	20	18 (90.0)	44	42 (95.5)
'Gemeiner D.'	8	8 (100)	20	4 (20.0)
'Golden Promise'	96	85 (88.5)	260	258 (99.2)
'Himalaya'	41	39 (95.1)	59	50 (84.7)
'Local sebergelon'	10	2 (20.0)	5	5 (100)
'Maris Mink'	35	35 (100)	38	37 (97.4)
'Midas'	29	23 (79.3)	62	42 (67.7)
'Oac Ottawa'	6	6 (100)	8	8 (100)
'Pfauengerste'	24	15 (62.5)	15	7 (46.7)
'Ricotense'	7	3 (42.9)	3	3 (100)
'Wisconsin'	12	3 (25.0)	7	6 (85.7)

Table 5. Somatic embryo and plant production from individual immature embryos of cv 'Golden Promise'. Embryogenic callus was induced on CC medium supplemented with 1 g/l casein hydrolysate and 2 mg/l picloram. The somatic embryos were transferred to CC medium with 1 mg/l IAA and 0.05 mg/l zeatin. (Data for plants regenerated after the first subculture cycle)

Immature embryo no.	No. of somatic embryos transferred to regeneration medium	No. of plantlets transferred to soil	Percentage of somatic embryos developing to plants
1	35	28	80.0
2	26	26	100
3	47	33	70.2
4	33	24	72.7
5	44	23	52.3
6	36	35	97.2
7	32	32	100
8	15	15	100
9	10	10	100
10	20	10	50.0
11	13	13	100
12	19	19	100
Mean (±SE):	27.5 (±12.2)	22.3 (±8.9)	85.2 (±19.5)

summers in Cologne were somewhat cold and rainy, whereas June and July of 1986 were extremely hot and dry. Thus, the importance of environmental factors on the quality and quantity of callus growth in the different seasons has to be considered.

Some examples of regeneration frequencies obtained in 1985 from calli transferred to regeneration media 4 weeks after culture initiation are presented in Table 4. In this experiment, only the plants regenerated during the first culture cycle were considered. Rather large portions of embryogenic calli were transferred to regeneration medium and clusters of shoots or multiply-tillering plantlets were planted in soil. While this precluded the exact counting of plants regenerated per explant, genotypic differences in regeneration frequency were nevertheless clearly demonstrated. In Table 4, each cluster of somatic embryos transferred to regeneration medium is classed as one regenerated plant. A more exact estimate of the number of somatic embryos produced per explant and of the number of plantlets per somatic embryo was obtained following the development of individual calli. Data from 12 such cultures are presented in Table 5.

The regeneration capacity of embryogenic cultures decreased during successive subcultures. Calli of genotypes Dissa and Oac Ottawa were maintained in an embryogenic state for 8 months. At the present time, seeds have been harvested from more than 800 direct regenerants of various genotypes.

Discussion

The present study demonstrates that in barley the frequency of somatic embryo formation and of plant regeneration is under genotypic control. Out of 36 different barley lines tested in the initial screening experiment, 21 formed friable callus and 15 of them also produced an embryogenic callus-type. Plant regeneration was achieved from eight of these lines. Similar genotype-dependent differences in the ability of embryogenic callus formation and plant regeneration have been described for various cereals. Numerous maize genotypes have been considered in in vitro genetic studies (Duncan et al. 1985; Tomes and Smith 1985; Hodges et al. 1986). While Hodges et al. (1986) suggested that one or two nuclear genes are important for somatic embryogenesis and regeneration capacity, the study by Tomes and Smith (1985) indicated significant negative maternal effects. In barley, Foroughi-Wehr et al. (1982) reported that in anther cultures the capacity for callus induction and plant regeneration can be inherited and is probably under the control of nuclear genes. Using immature embryos as explants, Hanzel et al. (1985) compared 91 barley genotypes and found that 45 formed callus and from eight lines plants could be regenerated.

In the last paper cited it was demonstrated that besides genotypic effects the composition of the culture media also influenced callus growth and totipotency. In our experiments a further increase in capacity and frequency of embryogenic callus induction was achieved by successive modifications of the media. It was found that 23 of 24 lines tested on improved media formed somatic embryos and regenerated to plants. Only the cultivar Jane Hadaka showed no morphogenic capacity on all media tested. In tissue culture experiments with barley, basal or modified MS medium was frequently adopted for callus induction and plant regeneration (Cheng and Smith 1975; Rengel and Jelaska 1986; Thomas and Scott 1985; Weigel and Hughes 1985). MS medium was often modified increasing the thiamine and inositol contents (Cheng and Smith 1975; Weigel and Hughes 1985), and our results confirm the benefit of these modifications. CC medium had never been previously tested for barley tissue culture, but positive results have been reported using basal (Orton 1979) or modified Gamborg B5 medium (Seguin-Swartz et al. 1984; Goldstein and Kronstadt 1986). The most common modification of B5 medium has been the addition of NH_4 to the basal salts. In our experiments, out of 36 lines tested only the winter-type line C143/82 produced embryogenic callus on Gamborg B5 medium.

In the present work it was demonstrated that in barley the frequency of embryogenic callus formation is increased by the addition of casein hydrolysate to the media. This mixture of amino acids where glutamin and proline prevail (Biester-Miel et al. 1985) has been used as a supplementary nitrogen source in other species (Duncan et al. 1985). Experiments with *Dactylis glomerata* L. have demonstrated the requirement for casein hydrolysate on somatic embryogenesis (Gray and Conger 1985). We obtained, however, negative results with the addition of amino acids due to the high concentrations tested. Positive roles of specific amino acids on quality of callus growth and the induction of somatic embryogenesis has been reported for maize (Armstrong and Green 1982) and for alfalfa (Stuart and Strickland 1984).

In cereals, the auxin 2,4-D is of common use for the induction of embryogenesis in in vitro cultures (Bright and Jones 1985). Other synthetic auxins, such as picloram and dicamba, have also been found effective for inducing callus in graminaceous species (Conger et al. 1982). In barley, we induced somatic embryogenesis by supplementing media with either 2,4-D, dicamba, picloram or 2,4,5-T. The successful use of 2,4,5-T for callus induction and plant regeneration was also reported for four barley lines by Rengel and Jelaska (1986).

To improve the germination frequency of somatic embryos we supplemented regeneration media with different combinations and concentrations of hormones. Cytokinins were not absolutely required for shoot development, but 1 mg/l IAA and 0.05 mg/l zeatin supported the germination of somatic embryos. Other cytokinins like BAP, kinetin and 2iP induced browning of barley callus, while zeatin or zeatin riboside had a positive influence. Plant regeneration from embryo-derived callus of *Hordeum spontaneum* and *Hordeum bulbosum,* has been also achieved with media containing IAA and zeatin (Breimann 1985).

Besides genotype and medium composition, we found that the physiological state of the donor plant which depends on the growth environment affected the reaction of the explants under in vitro conditions. Field-grown material was tested in 3 subsequent years. In 1985 and 1986 immature embryos were cultured on the same induction media. Nevertheless, frequencies of embryogenic callus induction differed for each cultivar in these 2 years. This observation reflects the different climatic conditions experienced in Cologne in the two summers (1985: cold and rainy; 1986: dry and hot). A relation between the physiological state of the donor material and the response of explants in culture was reported for maize by Santos and Torne (1986). They further observed that different growth conditions influenced each cultivar differently. In barley, Dale and Deambrogio (1979) suggested that immature embryos ranging in size from 0.7 to 1.4 mm, corresponding to 10 to 13 days after pollination, were best suited for obtaining vigorous callus induction. In contrast, Goldstein and Kronstadt (1986) reported that embryos excised from

plants grown under their greenhouse conditions (with higher temperatures) developed much faster. In their experiment, younger but also smaller embryos resulted in more suitable for callus induction. These data and our results suggest that each genotype has an optimal physiological stage of the explants which also depends on the growth environment of the donor plant. It may be possible to induce totipotent callus from a genotype previously unresponsive like cv Jane Hadaka by changing the growth conditions of the donor plant and/or adopting a different stage of the explant. The growth conditions being used routinely might not be optimal in all cases, but should rather be defined as acceptable working conditions.

For several barley genotypes we could establish an efficient and reproducible in vitro system for plant regeneration via somatic embryogenesis. This system can be used for in vitro selection studies. Embryogenic callus reveals, moreover, an important material for the initiation of embryogenic cell suspensions. However, an obstacle to the establishing of long-term embryogenic suspensions is the decline of totipotency of the callus cultures. In our most long-lived cultures plant regeneration capacity was lost 8 months after culture initiation. An efficient system for embryogenic callus induction may, in any case, provide enough material for re-establishing new suspensions. Homogeneous suspensions offer the possibility of isolating dividing protoplasts (Ozias-Akins and Lörz 1984; Lührs and Lörz 1986), which can be used for direct gene transfer studies (Lörz et al. 1985).

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