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Enhancement of plant regeneration from embryogenic callus of commercial barley cultivars

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Abstract Genotypic restrictions on plant regeneration from cultured cells have hindered the genetic transformation of most barley cultivars. Optimizing culturing protocols for specific cultivars of commercial interest may facilitate their genetic transformation. Plant regeneration from embryogenic callus of 'Harrington', 'Morex', and 'Hector' as affected by certain protocol modifications was examined in replicated experiments. Regeneration was improved for all cultivars by separately autoclaving certain components of the culture media and by reducing the amount of embryogenic callus cultured per petri dish. Regeneration improvements in response to various concentrations of copper and 2,4-dichlorophenoxyacetic acid were more genotype specific. This study suggests that the development and use of genotype-specific protocols can enhance plant regeneration. Enhancements in plant regeneration are expected to facilitate the transformation of commercial barley germplasm.

Key words Barley · Tissue culture · Plant regeneration · Copper

Abbreviations *2,4-D* 2,4-Dichlorophenoxyacetic acid · *SPDL* Single plant-derived line

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Introduction

The recovery of fertile plants from transgenic callus is a critical component of many cereal transformation systems. Microprojectile-mediated DNA delivery into immature embryo-derived callus or scutellar tissue of immature embryos and subsequent plant regeneration have resulted in transgenic rice, oat, wheat, and barley plants (Christou et al. 1991; Somers et al. 1992; Weeks et al. 1993; Wan and Lemaux 1994). However, efficient regeneration of plants from transformed embryogenic callus often is limited to specific genotypes that exhibit vigorous plant regeneration. For barley, the system as described by Wan and Lemaux (1994) is not an efficient method for the transformation of certain cultivars of commercial importance in North America, such as 'Harrington' and 'Morex' because few green plants can be recovered from transgenic tissues (P. G. Lemaux, personal communication; L. S. Dahleen, unpublished).

Specific modifications of the culture medium have been shown to influence plant regeneration from embryogenic barley callus. Bregitzer et al. (1995) reported that the regeneration of green plants was positively associated with 2,4-D concentration in three cultivars. Ziauddin and Kasha (1990a, b) also reported an influence of 2,4-D on plant regeneration from embryogenic barley callus, but their data suggested that the association was negative. Interactions between 2,4-D concentrations and the specific genotypes examined in the two studies may explain the apparently discrepant results.

Dahleen (1995) determined that increasing the copper concentration by as much as 50-fold that found in MS medium (Murashige and Skoog 1962) improved plant regeneration from two barley cultivars, 'Hector' and 'Excel'. The response was genotype-specific, with optimal concentrations for the two studied cultivars differing tenfold. The basis for this response is unknown, but it has been suggested that 1–25 μ*M* cupric sulfate in hydroponic nutrient solutions inhibits the formation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid in rice (Lidon

et al. 1995). Ethylene has been identified as a potent modulator of the in vitro response in a number of plant species, including barley (Biddington 1992; Cho and Kasha 1989; Evans and Batty 1994).

Media preparation methods may influence the availability of, or induce interactions between, certain nutritional components. Schenk et al. (1991) reviewed literature that linked carbohydrate breakdown induced by autoclaving to adverse effects on cultured plant cells. They demonstrated reductions in sucrose degradation and precipitate formation as a result of separating the iron-EDTA and potassium phosphate components from the other components in MS medium during autoclaving.

The experiments reported here were initiated to develop methods that enhance green plant regeneration from callus of two commercially important cultivars, 'Morex' and 'Harrington'. These cultivars are the current cultivar standards for malt quality as defined by the American Malting Barley Association (740 N Plankinton Rd, Suite 830, Milwaukee, WI, 53203 USA) and are representatives of important pools of North American six-rowed and two-rowed germplasm, respectively. Both cultivars have a history of relatively poor plant regeneration. In addition to these cultivars, 'Hector' was included to facilitate comparisons with the study of Dahleen (1995). 'Hector' is well-adapted to the dryland conditions common to the western plains of North America. It has been widely grown and used as a parent to produce newer cultivars; however, 'Hector' is being gradually replaced by agronomically superior cultivars.

The specific objectives of these experiments were to determine the effects on green plant regeneration of (1) media preparation methods, (2) the amount of embryogenic callus cultured per petri plate, and (3) the concentrations of 2,4-D and copper in the culture media.

Materials and methods

Explant-donor growth conditions

Single plant-derived lines (SPDLs), selected from breeders seed of each cultivar, were used as explant sources for all experiments. At least five individual plants from each SPDL were used for each experiment or replicate. For 'Morex', SPDL 2 was used based on its significantly superior regeneration compared to several other Morex SPDLs (P. Bregitzer, unpublished). The 'Harrington' and 'Hector' SPDLs were chosen randomly. Field observations and agronomic data indicate that these SPDLs are representative of their respective cultivars (P. Bregitzer and L. S. Dahleen, unpublished).

Experiments were conducted at two locations, Aberdeen, Idaho, and Fargo, North Dakota. At Aberdeen, donor plants were grown in a 0.42-m^2 growth chamber. Fourteen hours of light was provided daily by three 400-W high-intensity, high-pressure sodium bulbs and three 400-W high-intensity metal halide bulbs. Light:dark temperatures were 20°:10 °C with 1-h ramping intervals between temperatures. Two plants were grown per plastic pot (12×12×15 cm) containing approximately 2.0 l of a mix of sand:peat:vermiculite (3:2:2 ratio) supplemented with a commercially available slow-release blend of macro- and micronutrients. Biweekly additions of approximately 250 ml half-strength Hoagland's solution (Sigma Chemical, St. Louis, USA) were added when plants reached the boot stage.

At Fargo, donor plants were grown in a greenhouse at 20°–24 °C day and 13°–17 °C night temperatures. A daylength of 16 h was maintained by supplemental lighting with mercury halide lamps. One plant was grown per clay pot (15 cm in diameter by 18 cm depth) containing approximately 1.2 l of a soil-less mix of peat and vermiculite supplemented with a commercially available slow-release blend of macro- and micronutrients. No additional fertilizer supplementation was made.

Callus initiation and plant regeneration

Callus was initiated from immature zygotic embryos as described by Bregitzer et al. (1995). Five embryos (1–3 mm in length) were plated scutellum-down on 30 ml MS medium in a 15×100 mm petri plate (Aberdeen) or 50 ml MS medium in a 25×100 mm Petri plate (Fargo). The medium was solidified with 3.5 g l^{-1} Phytagel (Sigma[1]), modified as described by Bregitzer (1992), and supplemented with 3% maltose and 3 mg l^{-1} (6.8 μ*M*) 2,4-D. The method used to prepare the medium was modified by autoclaving separately the following components: (1) iron and potassium phosphate, (2) maltose, and (3) all other ingredients. These components were cooled to approximately 65 °C and then combined.

The developing calli were maintained in the dark (Fargo) or in dim light (Aberdeen – approximately 1 μmol m^{-2} s^{-1}) provided by shaded fluorescent lamps. Germinating zygotic meristems were removed from the developing calli 1–3 weeks after the embryos were placed on the medium (initiation date). Four weeks after initiation, embryogenic sectors from the three or four most vigorously growing calli were subcultured on MS medium prepared as above but with 2.5 mg l^{-1} (11.1 μ*M*) 6-benzylaminopurine and without 2,4-D supplementation. Calli were cultured on this medium for 1 week and then transferred to 25×100-mm petri plates containing 50 ml MS medium with no hormones (MSO); they were then incubated under moderate light (approximately 5 μmol m^{-2} s^{-1}) provided by fluorescent lamps. Following a culture period of 4 weeks on this medium, well-developed green plantlets (as described by Bregitzer et al. 1995) and any albino plantlets (including those that were poorly developed) were counted and discarded. Less well-developed green plantlets and green, shoot-like structures were transferred to half-strength MSO in 25×100-mm petri plates (Aberdeen) or Magenta boxes (Fargo) (Sigma) and cultured for an additional 4 weeks, at which time a final count of well-developed plantlets was made.

Specific modifications to this protocol were made as follows: (1) for comparisons of media preparation methods, all media components were combined and autoclaved in the "standard" treatment; (2) for comparisons of the amount of embryogenic callus cultured per plate, one to five embryos were cultured per petri plate, all callus was transferred to initiate plant regeneration, and callus fresh weights were measured at the time of transfer; (3) for comparison of 2,4-D and copper concentrations, various concentrations of these ingredients were added as specified in the results.

Statistical analyses

Each petri plate was considered to be an experimental unit. Data were collected on a per plate basis, and the number of plants per embryo and the percentage of plants that were green were calculated. Each replicate/genotype/treatment combination consisted of 12 plates. Experiments separated by time were considered as separate replicates. Experiments that generated non-normally distributed data that could not be satisfactorily transformed to permit linear analyses were analyzed with the CATMOD analysis within SAS (Statistical Analysis Systems, SAS 1988) This procedure provides chi square-based analyses of variance which cannot be used to generate multiple comparisons or confidence intervals. Normally distributed data were analyzed via the GLM procedure within SAS.

Table 1 Plant regeneration from callus of three barley genotypes in response to autoclaving method

Genotype[a]	Treatment	
	Standard autoclaving	Separate component autoclaving
Harrington	0.3/78[b,c]	0.8/78
Hector	1.2/90	2.1/83
Morex	0.7/48	1.2/66

[a] Each genotype is derived from a single plant selection of the cultivar
[b] Data are presented as: green plants per embryo/percentage of green plants
[c] Probability levels from CATMOD ANOVA. For green plants per embryo/percentage of green plants, respectively. Genotype: P = 0.001/NS; Treatment: P = 0.001/NS; G×T: P = 0.01/0.01

Table 2 Plant regeneration from callus of three barley genotypes in response to increasing amounts of callus

Number of embryos cultured per plate	Callus fresh weight (mg)[a]	Green plants per embryo[b]	Green plants per gram fresh weight[b]	Number of plants per plate
1	705	2.9	4.4	2.9
2	1229	2.0	3.5	4.0
3	1891	1.8	3.1	5.4
4	2322	1.3	2.6	5.2
5	2622	1.0	2.2	5.0

[a] Measured 4 weeks after callus initiation. The increase in callus fresh weight from one to four embryos was linear (P = 0.001)
[b] Probability levels from ANOVA: For green plants per embryo/percentage of green plants, respectively, P = 0.001/0.001

Results and discussion

Media preparation methods

This experiment consisted of four replicates and was conducted at Aberdeen. Media produced by co-autoclaving all ingredients was a light yellowish-brown, and a precipitate was noticed in medium without Phytagel (produced for observation only) when it was allowed to cool overnight. The media prepared with the modified, separate-component autoclaving technique did not form a precipitate and was essentially colorless.

Callus produced on both treatments was friable and yellowish-white and grew vigorously. Plant regeneration in response to culture on the standard versus the modified media is shown in Table 1. Analyses of variance using CATMOD indicated significant effects of genotype (P = 0.001), treatment (P = 0.001), and their interaction (P = 0.01) for green plants per embryo. This interaction arose from differences in the magnitude of treatment effects among genotypes, not from differences in the ranking of treatment effects, and thus for all genotypes the separate-component autoclaving technique was superior. Separating the iron and potassium phosphate components, and the maltose or sucrose component, from each other and from the other components of MS medium increased green plant regeneration 1.8 to 2.8 fold.

For the percentage of green plants, only the genotype× treatment interaction was significant (P = 0.01). Separate-component autoclaving increased the percentage of green plants from 'Morex' callus. The small decrease in the percentage of green plants from 'Hector' callus was not statistically significant.

Amount of callus cultured per petri plate

This experiment consisted of four replicates for 'Morex' and two replicates for 'Harrington' and 'Hector' and was conducted at Aberdeen. All treatments produced friable, yellowish-white, and vigorously growing callus. Analyses of variance using CATMOD indicated significant effects (P = 0.001) of genotype and the number of cultured embryos for both the number of green plants per embryo and the number of green plants per gram fresh weight. The genotype-by-embryo number interactions were not significant. Data for callus fresh weight and plant regeneration are shown in Table 2.

Analysis of variance using GLM indicated that callus fresh weight increased linearly (P = 0.001) when from one to four embryos were cultured per petri plate. Culturing five embryos per petri plate appeared to introduce limitations to callus growth, and the increase in callus fresh weight was no longer linear. The total number of green plants recovered per petri plate did not appear to increase in a linear fashion. The numbers of green plants recovered per embryo and the numbers of green plants recovered per gram of callus were negatively associated with the amount of callus cultured per plate.

It is not surprising that a negative relationship was seen between the number of green plants recovered per gram of callus and the number of embryos cultured per plate. However, the lack of correlation of callus growth rate and morphology with regeneration efficiency was surprising. These data indicate that callus morphology and growth rate are less sensitive to overcrowding than is plant regeneration. This has important implications for the recovery of plants from transgenic callus. If it is assumed that the number of totipotent cells per gram of callus does not vary with the amount of callus per plate, then maximizing the chance that any given transgenic cell will regenerate requires minimizing competition from other cells.

Optimization of copper and 2,4-D concentrations

This experiment consisted of two replicates at each of two locations (Aberdeen and Fargo). All treatment combinations produced friable, yellowish-white, and vigorously growing callus. There were no consistent differences attributable to location, and interactions with location were considered as part of the experimental error. Analyses of variance using CATMOD indicated that the number of

Table 3 Plant regeneration from callus of three barley genotypes in response to various concentrations of copper and 2,4-D, respectively

Treatment	Genotype [a]		
	Harrington	Hector	Morex
0.1 μ*M* Cu	0.9/91 [b,c]	2.8/90	2.8/73
5.0 μ*M* Cu	1.9/93	5.4/93	5.9/79
50 μ*M* Cu	2.2/97	5.1/91	5.4/74
3 mg l^{-1} 2,4-D (13.6 μ*M*)	1.5/95	4.5/93	5.3/75
4.5 mg l^{-1} 2,4-D (20.4 μ*M*)	1.6/93	4.2/92	4.5/76
6 mg l^{-1} 2,4-D (27.1 μ*M*)	1.8/93	4.5/88	4.3/76

[a] Each genotype is derived from a single plant selection of the cultivar
[b] Data is presented as: green plants per embryo/percentage of green plants
[c] Probability levels from CATMOD ANOVA. For green plants per embryo/percentage of green plants, respectively. Genotype: P = 0.001/0.001; copper concentration: P = 0.001/0.001; 2,4-D concentration: P = 0.01/NS; genotype×copper: P = 0.001/0.001; genotype× 2,4-D: P = 0.001/0.001; all other interactions: non-significant

Table 4 Plant regeneration from callus of 'Morex' [a] in response to various concentrations of copper and to autoclaving method

Copper concentration	Autoclaving method	Green plants per embryo	Percentage green plants
0.1 μ*M*	Standard	0.7 [b]	48
0.1 μ*M*	Modified	6.2	58
5.0 μ*M*	Standard	2.5	58
5.0 μ*M*	Modified	7.3	66

[a] The genotype tested was derived from a single plant selection from 'Morex'
[b] Probability levels from CATMOD ANOVA for treatment: P = 0.001 and 0.004 for green plants and percentage green plants, respectively

green plants per embryo was significantly influenced by genotype (P = 0.001), the concentrations of copper (P = 0.001) and 2,4-D (P = 0.01), and the interactions of genotype with the concentrations of copper (P = 0.001) and with 2,4-D (P = 0.01). These sources of variance, except for 2,4-D concentration, were significant also for the percentage of green plants. The interaction between copper and 2,4-D concentrations was not significant, and the mean regeneration responses are presented as main effects of copper and 2,4-D concentrations in Table 3.

Elevated copper concentrations increased the number of green plants per embryo from 1.9 to 2.4 fold and slightly increased the percentage of green plants. For 'Morex' and 'Hector', 5 μ*M* appeared to be optimal, and 'Harrington' responded best to 50 μ*M*. The response to 2,4-D was less clear, with the number of green plants per embryo positively associated with 2,4-D concentration for 'Harrington' but negatively associated for 'Morex'.

Additional experiments were conducted to determine if the optimal concentration of 2,4-D for 'Morex' was less than 3 mg l^{-1} on media containing 5 μ*M* copper. The numbers of green plants per embryo on media containing 3, 1.5, and 0.75 mg l^{-1} 2,4-D were 2.7, 2.6, and 1.7. Thus, 3 mg l^{-1} 2,4-D appeared to be optimal for 'Morex'.

Plant regeneration and 2,4-D concentration showed neither the clearly positive relationship seen by Bregitzer et al. (1995) nor the negative relationship seen by Ziauddin and Kasha (1990 a, b). Rather, a genotype-specific, nonlinear response was seen. Such a response may explain the differing conclusions reached by these authors and illustrates the potential importance of developing genotype-specific protocols for callus growth and plant regeneration.

Response to optimized copper concentration and autoclaving method

This experiment consisted of two replicates at each of two locations (Aberdeen and Fargo) and tested the regeneration response of 'Morex'. The treatments were 0.1 or 5.0 μ*M* copper, and the standard or modified autoclaving method in a 2×2 factorial design. Data on the regeneration responses are shown in Table 4.

Analysis of variance using CATMOD indicated that there were significant treatment effects for the number and percentage of green plants (P = 0.001 and P = 0.004, respectively); interactions were not significant except as noted below. As noted in previous experiments, increasing copper levels or modifying autoclaving techniques improved the regeneration response; these effects were significant at both locations. For the percentage of green plants, the best treatment combination was high copper and modified autoclaving. This treatment combination also produced the greatest number of green plants; however, there was a location x treatment interaction. At Aberdeen, this treatment combination produced approximately 2.6-fold more plants than the next best treatment (5 μ*M* copper and coautoclaving). At Fargo the difference was less (1.1 fold), and was not statistically significant (P = 0.4). Nevertheless, the reduction in albinism in response to high copper combined with the modified autoclaving technique justifies a general recommendation of this treatment combination.

General comments

These studies clearly indicated that several components of our standard tissue culture protocol for barley callus were not optimal for any of the cultivars studied. Although the data from this and other studies do indicate that an optimal protocol must be developed on a genotype-specific basis, they also indicate that certain modifications may be generally applicable to cultivars with diverse pedigrees. Based on these data, we suggest that standard media for short-term barley callus culture should contain 5 μ*M* copper, that as little tissue be maintained per petri plate as practical, and that the carbon source and iron source should not be autoclaved with other media components. The optimal concentration of 2,4-D will be genotype-specific, but 3 mg l^{-1} may be adequate for most cultivars.

These modifications to optimize plant regeneration from barley callus will be most useful in the context of re-

covering plants from transgenic barley tissues. The use of elevated copper levels has been found to greatly facilitate the recovery of transgenic, green barley plants from a number of previously recalcitrant cultivars, including 'Harrington' (P. G. Lemaux et al., personal communication). It is likely that some or all of the other modifications suggested by these studies can be used to provide additional improvements in the regeneration of plants from transgenic barley callus.

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References

Biddington NL (1992) The influence of ethylene in plant tissue culture. Plant Growth Reg 11: 172–187

Bregitzer P (1992) Plant regeneration and callus type in barley: effects of genotype and culture medium. Crop Sci 32: 1108–1112

Bregitzer P, Campbell RD, Wu Y (1995) Plant regeneration from barley callus: effects of 2,4-dichlorphenoxyacetic acid and phenylacetic acid. Plant Cell Tissue Organ Cult 43: 229–235

Cho U-H, Kasha KJ (1989) Ethylene production and embryogenesis from anther cultures of barley (*Hordeum vulgare* L.). Plant Cell Rep 8: 415–417

Christou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Biotechnology 9: 957–962

Dahleen LS (1995) Improved plant regeneration from barley callus cultures by increased copper levels. Plant Cell Tissue Organ Cult 43: 267–269

Evans JM, Batty NP (1994) Ethylene precursors and antagonists increase embryogenesis of *Hordeum vulgare* L. anther culture. Plant Cell Rep 13: 676–678

Lidon F Cm, Da Graca Barreiro M, Santos Henriques F (1995) Interactions between biomass production and ethylene biosynthesis in copper-treated rice. J Plant Nut 18: 1301–1314

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

SAS (1988) SAS/STAT user's guide. Release 6.03 edn. SAS, Cary, N.C.

Schenk N, Hsiao K-C, Bornman CH (1991) Avoidance of precipitation and carbohydrate breakdown in autoclaved plant tissue culture media. Plant Cell Rep 10: 115–119

Somers DA, Rines HW, Gu W, Kaeppler HF, Bushnell WR (1992) Fertile, transgenic oat plants. Biotechnology 10: 1589–1594

Wan Y, Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. Plant Physiol 104: 37–48

Weeks JT, Anderson OD, Blechl AE (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum* L.). Plant Physiol 102: 1077–1084

Ziauddin A, Kasha KJ (1990a) Long-term callus cultures of diploid barley (*Hordeum vulgare* L.). I. Auxin effects on culture initiation and maintenance. Euphytica 48: 171–176

Ziauddin A, Kasha KJ (1990b) Long-term callus cultures of diploid barley (*Hordeum vulgare* L.). II. Effect of auxins on chromosomal status of cultures and regeneration of plants. Euphytica 48: 279–286