

Optimization of media nitrogen and copper concentrations for regeneration of green plants from polyembryogenic cultures of barley (*Hordeum vulgare* L.)

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Abstract

In recent years particle bombardment has become the most used method for gene transfer to barley (*Hordeum vulgare* L.). Transformation efficiency depends greatly on the ability of the target material to regenerate into green plants. In this work we improved the regeneration efficiency of polyembryogenic cultures of barley (cv. Kymppi) by optimizing the nitrogen and copper concentrations in the media. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Barley; *Hordeum vulgare*; Mathematical models; Medium optimization; Polyembryogenesis; Regeneration

1. Introduction

Immature embryos and polyembryogenic cell mass of barley (*Hordeum vulgare* L.) have often been used as target material for gene transfer by microprojectile bombardment [1–3]. The transformation efficiency depends greatly on the ability of the target material to regenerate into green plants. Although acceptable regeneration frequencies have been achieved, albinism has been a problem. The choice of culture medium affects regeneration efficiency and the proportion of green plants obtained. Previous studies have clearly demonstrated that the nitrogen balance of the culture medium has a major effect on the somatic embryogenesis of several species. Especially the nitrate:

ammonium ratio and the addition of reduced nitrogen in the form of amino acids affect somatic embryogenesis [4–8]. Furthermore, the nitrogen requirements vary between different stages of embryogenesis. This might be due to differences in the endogenous amino acid metabolism during morphogenetic processes such as embryogenesis and germination. Recently Higashi et al. [9] reported clear time course changes in glutamine synthase (GS) activity during carrot somatic embryogenesis

The media used for barley anther culture have been optimized with respect to their nitrogen concentration by several groups [10–12]. However, the nitrogen compositions of media used for polyembryogenic cultures induced from immature embryos have never been systematically optimized. In the present study we were able to enhance the regeneration efficiency of polyembryogenic cultures of barley cv. Kymppi by optimizing the nitrogen composition of the culture media. We were also able to improve the regeneration by modifying the copper content of the medium.

Abbreviations: GS, glutamine synthase; 2,4-D, 2,4-dichlorophenoxy acetic acid.

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2. Materials and methods

2.1. Plant material

Barley (*H. vulgare* L. cv Kymppi) plants were grown in a climate chamber under a 19 h light/5 h dark cycle with a day temperature of 22°C and a night temperature of 13°C and 40–50% relative humidity. Light levels at head height were $\approx 250\text{--}300 \mu\text{M m}^{-2}\text{s}^{-1}$. Plants were potted in soil mix (Vermiculite:peat:soil; 2:1:1) and fertilized weekly with Kukkien Y-lannos (Maatalouspalvelu Oy, Finland), Biolan S.M.3 (Biolan Oy, Finland) and Puutarhan täyslannos (Kemira Oy, Finland) according to the manufacturer's instructions. Immature seeds of cv Kymppi with embryos about 1–1.5 mm in size were collected. The seeds were surface sterilized with 70% ethanol for 5 min and with sodium hypochlorite (4% available chlorine) for 10 min and thoroughly rinsed with sterile water. The immature embryos were dissected and the embryonic axis was removed with a surgical

blade. The isolated scutella were placed either on the original polyembryogenesis medium or on an experimental medium according to the experimental plan.

2.2. Polyembryogenesis medium

The isolated scutella were cultivated on a solid L2 medium [13], which was modified by reducing the 2,4-D concentration to 1 mg l^{-1} . In the optimization experiments the amounts of ammonium, nitrate and organic nitrogen were altered according to the experimental design (Table 1). The ratio of organic components (glutamine:proline:asparagine; 7:1:1) in the media was kept constant.

2.3. Regeneration medium

For regeneration the cell cultures were placed on modified MS medium [14–16] containing 0.4 mg l^{-1} BA, 35 g l^{-1} maltose and 3 g l^{-1} gellan

Table 1
Optimization of polyembryogenesis and regeneration media^a

Experiment	Coded values			Real values: polyembryogenesis medium (mM)			Real values: regeneration medium (mM)		
	x_1^b	x_2^c	x_3^d	X_1	X_2	X_3	X_1	X_2	X_3
1	-1	-1	-1	15	8	6	11	2	4.5
2	1	-1	-1	45	8	6	31	2	4.5
3	-1	1	-1	15	28	6	11	6	4.5
4	1	1	-1	45	28	6	31	6	4.5
5	-1	-1	1	15	8	20	11	2	15.5
6	1	-1	1	45	8	20	31	2	15.5
7	-1	1	1	15	28	20	11	6	15.5
8	1	1	1	45	28	20	31	6	15.5
9	-1.682	0	0	4.8	18	13	4.2	4	10
10	1.682	0	0	55.2	18	13	37.8	4	10
11	0	-1.682	0	30	1.2	13	21	0.6	10
12	0	1.682	0	30	34.8	13	21	7.4	10
13	0	0	-1.682	30	18	1.2	21	4	0.7
14	0	0	1.682	30	18	24.8	21	4	19.3
15	0	0	0	30	18	13	21	4	10
16	0	0	0	30	18	13	21	4	10
17	0	0	0	30	18	13	21	4	10
18	0	0	0	30	18	13	21	4	10
19	0	0	0	30	18	13	21	4	10
20	0	0	0	30	18	13	21	4	10

^a Experimental design and its independent variables.

^b X_1 , nitrate (mM).

^c X_2 , ammonium (mM).

^d X_3 , organic nitrogen (mM).

gum (Phytigel, Sigma). In the optimization experiments the amounts of ammonium, nitrate and organic nitrogen (glutamine) were altered according to the experimental design (Table 1).

2.4. Experimental plan: polyembryogenesis

For optimization of polyembryogenesis medium, isolated scutella from five immature seeds were placed on each experimental plate. Each experimental polyembryogenesis medium plate was considered as a separate experimental unit. The plates were cultivated in the dark at 22°C. After 2 weeks the polyembryogenic mass was subcultured on regeneration medium. Regeneration medium was kept constant during optimization of the polyembryogenesis medium. The plates were cultivated under a 16 h light/8 h dark cycle at 22°C and the regenerants were recorded every 2 weeks for 1.5 months. The optimized polyembryogenesis medium was compared with the original polyembryogenesis medium in a separate experiment. The regeneration medium was also kept constant in this experiment.

2.5. Experimental plan: regeneration

For optimization of regeneration medium, sieved cell mass was used to ensure uniform inoculum at the regeneration stage. Ten isolated scutella were placed on each original polyembryogenesis medium plate. After 1 week the polyembryogenic cell mass from 350 scutella was suspended in 100 ml of liquid polyembryogenesis medium. The suspension was agitated on a horizontal rotary shaker (Infors AG, 133 rpm, stroke radius 2.5 cm) for 1.5 h. The resulting suspension was sieved and the cells passing through the sieve (1000 µm pore diameter) were collected by centrifugation (300 × g; 5 min). The centrifuged cells were diluted with medium to 3 ml. Fresh polyembryogenesis medium plates were inoculated with 0.25 ml each of this suspension. The plates were cultivated in the dark at 22°C. After 2 weeks the polyembryogenic mass was subcultured on experimental regeneration media. The mass produced on each polyembryogenesis medium plate was considered as a separate experimental unit. Polyembryogenesis medium was kept constant during the optimization of regeneration medium. The plates were cultivated under a 16 h light/8 h dark cycle at

22°C and the regenerants were recorded every 2 weeks for 1.5 months. The optimized regeneration medium was compared with the original regeneration medium in a separate experiment. The polyembryogenesis medium was also kept constant in this experiment. A medium with no organic nitrogen was included in the experiment, because the optimal concentration was the lowest organic nitrogen level tested.

2.6. Experimental design and mathematical methods

Ammonium, nitrate and organic nitrogen concentrations were chosen as independent variables for optimization of polyembryogenesis and regeneration media, employing a 2³-factorial experimental design with six star points and six replicates at the center point (Table 1) [17,18]. The total number of experiments was 20. Second order polynomial mathematical models describing the effects of selected variables on the number of green, albino and total plantlets regenerated were derived from the experimental results.

2.7. Optimization of the copper concentration

The amount of copper in the media was optimized stepwise. Both the polyembryogenesis and regeneration media were supplemented with 1, 5 or 10 µM copper sulfate. The concentration of copper in the original media was 0.1 µM. Five scutella were placed on polyembryogenesis medium and cultivated in the dark at 22°C. After 2 weeks the cell mass was transferred to regeneration medium with the same copper concentration as in the polyembryogenesis medium. The plates were cultivated under a 16 h light/8 h dark regime at 22°C and the regenerants were recorded every 2 weeks for 1.5 months.

3. Results

3.1. Polyembryogenesis medium

The highest number of green plants was obtained with low nitrate (4.8 mM), low ammonium (1.2 mM) and high organic nitrogen concentrations (24.8 mM) (Fig. 1). The highest number of total plants was also achieved with these concen-

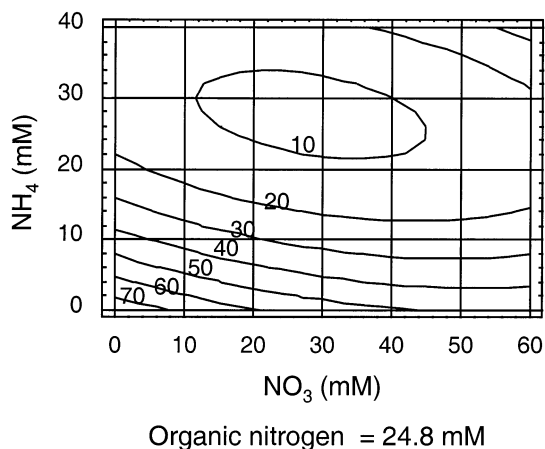


Fig. 1. Production of green barley plants (Y_1 , number of green regenerants/five scutella) as a function of nitrate (x_1), ammonium (x_2) and organic nitrogen (x_3) concentrations in polyembryogenesis medium: $Y_1 = 14.663 - 0.958x_1 - 1.773x_2 + 3.506x_3 + 0.009x_1^2 + 0.018x_1x_2 - 0.002x_1x_3 + 0.063x_2^2 - 0.089x_2x_3 - 0.039x_3^2$, $R^2 = 0.954^{***}$ ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$).

Table 2
Comparison of original and optimized polyembryogenesis media.

	Original medium	Optimized medium
Total nitrogen (mM)	67.9	30.8
Nitrate (mM)	36.1	4.8
Ammonium (mM)	18.7	1.2
Organic nitrogen (mM)	13.1	24.8
$\text{NO}_3:\text{NH}_4$	2:1	4:1
Inorganic:organic nitrogen	4:1	1:4
Green plants*	41 ± 16.8	45 ± 10.8
Albino plants*	2 ± 1.3	3 ± 2.2
Total plants*	42 ± 15.8	48 ± 9.0

* Average of four replicas \pm S.D.

trations. The differences between the optimal concentrations and those in the original medium are presented in Table 2. The production of albinos cannot be reliably predicted from the equation. However, Fig. 2 shows that albino production was very low at the optimal area for green plant production.

The results of the comparison of the original and the optimized polyembryogenesis media are shown in Fig. 3 and Table 2. The production of green plants was only slightly higher on the optimized medium than on the original medium but the standard deviation of the green plant produc-

tion was clearly smaller for the optimized medium. The total nitrogen concentration in the optimized medium was only about half the concentration in the original medium. The organic:inorganic nitrogen ratio was altered from the original 4:1 to 1:4.

3.2. Regeneration medium

According to Fig. 4, the optimal green plant production was achieved on a low organic nitrogen medium with high nitrate (37.8 mM) and 3 mM ammonium. Fig. 5 shows that the highest albino production was achieved on a high ammonium, low nitrate and low organic nitrogen medium. The lowest albino production was achieved with the same concentrations, which

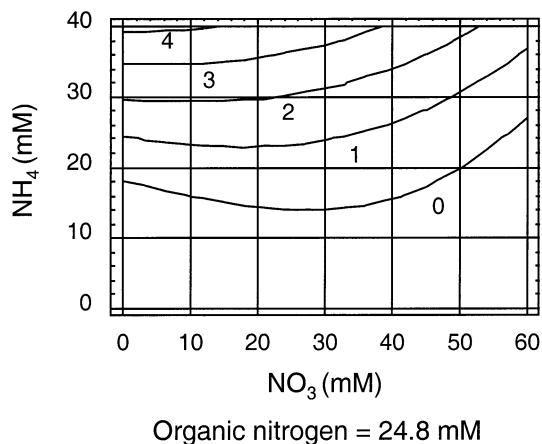


Fig. 2. Production of albino barley plants (Y_2 , number of albino regenerants/five scutella) as a function of nitrate (x_1), ammonium (x_2) and organic nitrogen (x_3) concentrations in polyembryogenesis medium: $Y_2 = -0.251 + 0.065x_1 - 0.096x_2 + 0.188x_3 - 0.001x_1^2 - 0.002x_1x_2 + 0.002x_2^2 + 0.007x_2x_3 - 0.011x_3^2$, $R^2 = 0.462$ NS.

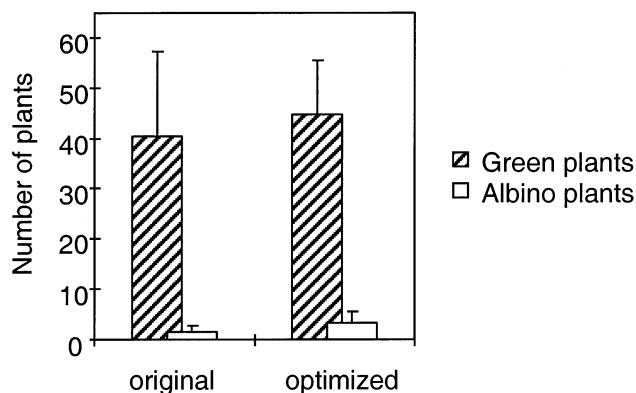


Fig. 3. Production of green and albino plants: comparison of the original and the optimized polyembryogenesis media.

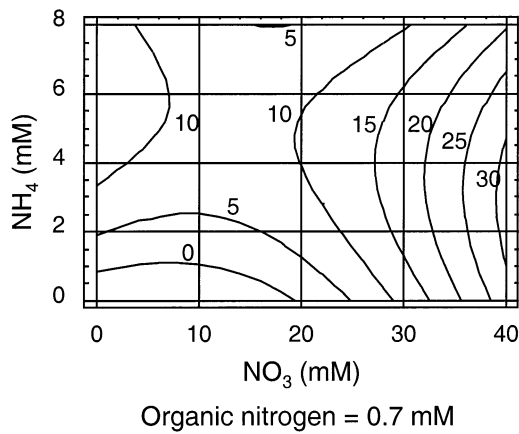


Fig. 4. Production of green barley plants (Y_3 , number of green regenerants/five scutella) as a function of nitrate (x_1), ammonium (x_2) and organic nitrogen (x_3) concentrations in regeneration medium: $Y_3 = -4.711 - 0.289x_1 + 5.974x_2 + 0.216x_3 + 0.028x_1^2 - 0.081x_1x_2 - 0.034x_1x_3 - 0.457x_2^2 - 0.148x_2x_3 + 0.034x_3^2$, $R^2 = 0.854^*$ ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$).

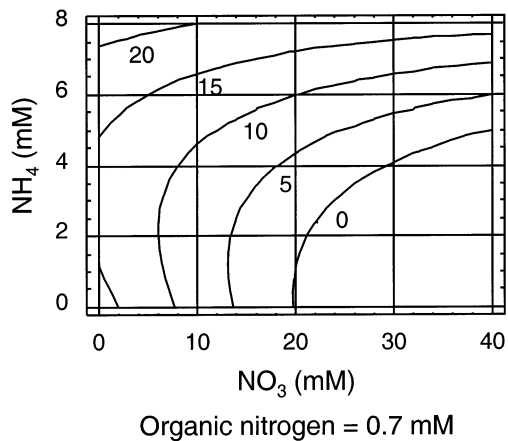


Fig. 5. Production of albino barley plants (Y_4 , number of albino regenerants/five scutella) as a function of nitrate (x_1), ammonium (x_2) and organic nitrogen (x_3) concentrations in regeneration medium: $Y_4 = 16.677 - 0.903x_1 - 1.750x_2 + 0.097x_3 + 0.002x_1^2 + 0.085x_1x_2 + 0.022x_1x_3 + 0.317x_2^2 - 0.200x_2x_3 + 0.002x_3^2$, $R^2 = 0.871^*$ ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$).

yielded the highest green plant production. The highest number of total plants was produced with low organic nitrogen (0.7 mM), high nitrate (40 mM) and high ammonium (8 mM) concentrations. However, in this area a considerable percentage of plants were albinos. The differences between the original medium and the optimized medium are presented in Table 3 and in Fig. 6.

The results of the comparison of the optimized medium with the original medium and the medium

with no organic nitrogen are shown in Fig. 6 and Table 3. The green plant production was clearly higher on the optimized medium than either of the other media. The albino production was also lowest on this medium. The total nitrogen concentration was increased in the optimized medium, as a result of the increased amount of inorganic nitrogen. The inorganic:organic ratio was drastically changed to 58:1 from the original 2:1. The concentration of organic nitrogen was only 0.7 mM in the optimized medium. However, if organic nitrogen was totally omitted from the medium, plantlet production was clearly reduced as can be seen in Fig. 6.

Table 3

Comparison of original and optimized regeneration media

	Original medium	Optimized medium
Total nitrogen (mM)	33.2	41.5
Nitrate (mM)	20.9	37.8
Ammonium (mM)	2.1	3
Organic nitrogen (mM)	10.3	0.7
NO ₃ :NH ₄	10:1	13:1
Inorganic:organic nitrogen	2:1	58:1
Green plants*	23 ± 6.2	42 ± 13.6
Albino plants*	2 ± 1.2	1 ± 0.6
Total plants*	25 ± 6.5	44 ± 13.1

* = average of 4 replicas ± S.D.

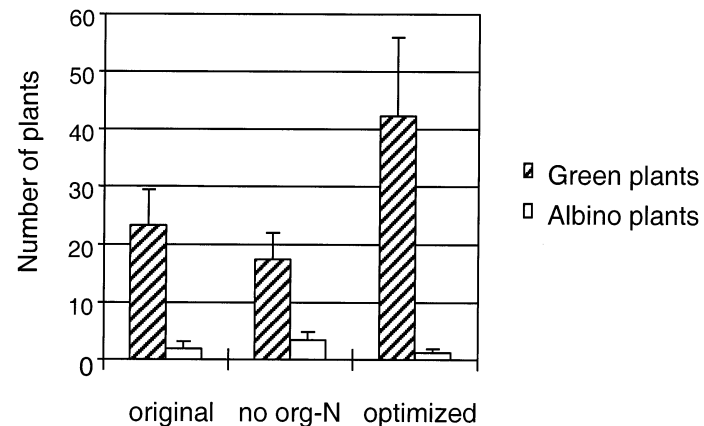


Fig. 6. Production of green and albino plants: comparison of the original and the optimized regeneration media and a regeneration medium without organic nitrogen.

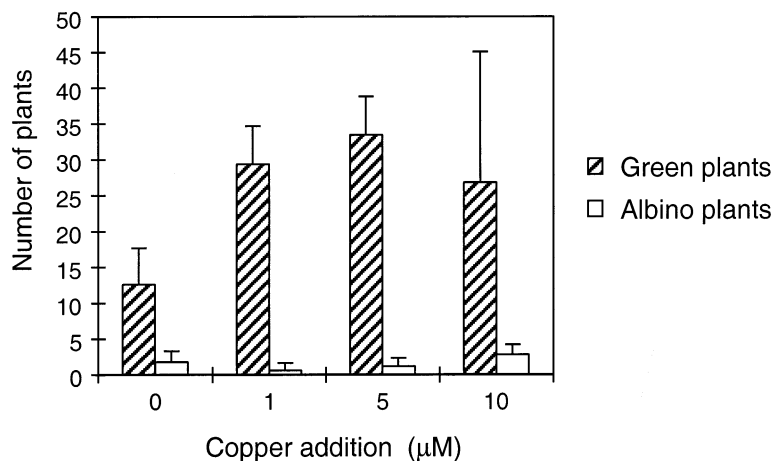


Fig. 7. Production of green and albino plants: the effect of copper addition into the media.

3.3. Effect of copper concentration

The effect of copper addition to the media is shown in Fig. 7. The highest green plant production with the lowest standard deviation was achieved with addition of 5 μM of copper into the media. At the highest level tested (10 μM) the copper began to have adverse effects on the production of green plants.

4. Discussion

Albinism continues to be a common problem in barley tissue culture, even though acceptable regeneration frequencies have been obtained for several elite barley cultivars [2,19,20]. From previous studies it is evident that albinism is a genotype dependent problem [19–21]. For some genotypes up to 30–50% of the regenerants have been albinos [12,19]. We demonstrated that both the nitrogen composition and the copper concentration have clear effects on the regeneration efficiency of polyembryogenic cultures of barley. By optimizing these factors we were able to improve the regeneration efficiency and also to reduce the proportion of albino regenerants in polyembryogenic cultures of barley cv. Kymppi.

It has recently been shown that the concentration of copper in MS media is not optimal for cereals: elevated levels of copper improved regeneration both in wheat [22,23] and in barley [24]. The results obtained in this work with the Finnish elite-cultivars Kymppi are consistent with these previous results. Dahleen [24] suggested that the

optimal concentration of copper for barley cultures is probably genotype dependent. Our results are in agreement with this suggestion. The optimal concentration for Kymppi was 5.1 μM (5 μM copper addition), which is clearly lower than the optimum found for the North American cultivator Hector (50 μM) but similar to the optimum found for the cultivator Excel (5 μM) [24].

The nitrogen composition of the medium has a clear effect on the regeneration of green plants from barley cell cultures. However, our results suggest that the nitrogen composition of the polyembryogenesis medium is not as crucial as the composition of the regeneration medium. This result is consistent with those of Mordhorst and Lörz [11], who found that when the nitrogen composition of media used for barley anther culture was altered the plant regeneration was strongly affected, whereas the other culture stages were only moderately influenced. In this study we were able to reduce the percentage of albino regenerants from 8.0 to 2.3% by optimizing the regeneration medium.

The polyembryogenesis stage clearly benefited from the addition of organic nitrogen. The total amount of nitrogen in the optimal medium was reduced but the proportion of organic nitrogen increased. Addition of organic nitrogen to embryogenesis medium in the form of different amino acids has been shown to enhance differentiation and somatic embryogenesis in barley [10,12,25] as well as in maize [26] and carrot [9] cell cultures.

In the optimized regeneration medium the amount of organic nitrogen and the ratio of or-

ganic to inorganic nitrogen were the factors that differed the most from the original medium. In the optimized medium the amount of organic nitrogen was low (0.7 mM) and the ratio of inorganic:organic nitrogen was 58:1. However, if organic nitrogen was totally omitted from the medium, the regeneration frequency decreased. Mordhorst and Lörz [11] found that an inorganic:organic nitrogen ratio of 90:10 gave the best regeneration frequency. Ratios of 100:0 and 71:29 gave clearly lower regeneration frequencies. Olsen [10] also found that omitting organic nitrogen from the medium caused the regeneration frequency of green plantlets to decrease. These results are consistent with those obtained in the present work.

In this study we found clear differences in the requirements of organic nitrogen between the polyembryogenesis and regeneration culture stages. These differences are most probably due to the developmental programming of plant embryos. Ohkawa and Maeda [27] reported that the major nitrogenous compound in developing endosperm of *Brassica napus* is glutamine, not ammonium ion. It has also been shown that the expression of glutamine synthase (GS) decreases strongly during maturation of embryos in maize [28] and in carrot [9]. In plantlets with mature chloroplasts, chloroplastic GS expression is tightly positively regulated by light [29]. These reports suggest that the early stages of embryogenesis require the presence of glutamine or other amino acids, which can be readily transformed to other amino acids and incorporated into proteins. In tissue culture this can be accomplished by adding organic nitrogen into the medium, as found in the optimization of the polyembryogenesis medium. At the maturation and regeneration stages the need for organic nitrogen supplementation decreases and in the green regenerants the chloroplastic GS is expressed and used for nitrogen assimilation.

Acknowledgements

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