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Effects of media components and environmental factors on shoot formation from protoplast-derived calli of *Solanum tuberosum*

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Abstract. The influence of several media components and environmental factors on shoot formation in protoplast-derived calli of *Solanum tuberosum* (a Rosamunda cross) were studied. Low sucrose concentration (3–15 mM) was beneficial for optimal shoot induction. Several concentrations of NO_3^- and NH_4^+ were suitable for shoot induction as long as the concentration of NO_3^- was about twice the concentration of NH_4^+ or higher. No stimulatory effect of glutamine, proline, putrescine, spermidine, spermine or adenine sulphate at 0.5 and 2 mM were found. White light promoted shoot induction compared with red and blue light or darkness. The intensity of light was shown to be a critical factor for good shoot induction. Lower light intensity ($30\,\mu E\,m^{-2}s^{-1}$) resulted in doubling of the number of calli producing shoots compared with higher ($110\,\mu E\,m^{-2}s^{-1}$) light intensity. A temperature of 20 °C promoted shoot regeneration compared to 25 °C. Based on these results improved conditions for regeneration of *S. tuberosum* are suggested, and shown to enhance shoot formation in five other genotypes tested.

Introduction

Reliable regeneration of plants from protoplasts is essential for the application of protoplast techniques in plant breeding, and more systematic knowledge on regeneration from protoplast-derived cell cultures is needed. Identification of critical factors in the cultivation process for one cultivar may also suggest favourable conditions for other cultivars or related species.

In the experiments presented here we studied shoot formation in protoplast-derived calli of a *Solanum tuberosum* breeding line (Beate × Maris Piper) × Rosamunda. The effects of growth-regulating substances

such as cytokinins and auxins as well as some lesser used growth-regulating factors such as polyamines and adenine were studied. Polyamines are involved in cell division and may also mediate the effects of plant hormones [4]. The concentration of sucrose plus mannitol in the medium have been varied extensively for *Solanum* species, but usually were kept between 90 and 200 mM [5, 11, 13]. In the present study, concentrations also outside this range were tested. Day length, light quality and intensity influence plant morphogenesis in vitro [14]. Some basic light treatments were included in the investigation. Finally the adjusted culture conditions were tested for 5 other potato genotypes of various origin.

Materials and methods

Plant material was provided by the Department of Crop Science, Agricultural University of Norway. One true seedling from the cross (Beate × Maris Piper) × Rosamunda was propagated by nodal cuttings (later referred to as × Rosamunda). Shoot cultures were grown on half-strength modified MS-medium, i.e. the TM5-medium of Shahin [12] with 1% sucrose and no growth regulators. The shoot cultures were grown in 350 ml Magenta boxes, kept at 20 °C and 110 $\mu E\,m^{-2}s^{-1}$, 12 h daylight, and transferred to fresh medium every month. The improved conditions were tested with *Solanum tuberosum* cv. Beate (commercial cultivar highly resistant to common scab), the pollen sterile breeding line N-80-34-37 (originating from *S. stoloniferum* × *S. tuberosum* backcrossed several times to *S. tuberosum* and crossed with Troll × Amve) (nematode-resistant, highly resistant to gangrene), the breeding line F × Aq (Falke × Aquila) (good resistance to light blight), and the breeding line N-73-20-262 (Troll × Amva) (highly resistant to gangrene). The commercial cultivar Beate was propagated as shoot culture originally derived from meristem culture. The other lines tested were propagated each from one tuber. A first-year seedling from the cross Heidrun × Ruth (later referred to as × Ruth) was also used.

Three to four-week-old in vitro grown shoots were used for protoplast isolation. Plant material was cut with a pair of scissors and exposed to a preconditioning medium for 6 h with 10% sucrose. vitamines, and half the concentrations of minerals as used for the plating medium [8]. The plant material was than incubated 12–14 h in an enzyme solution with 0.1% Macerozyme and 0.15% Cellulase R-10 [8]. Protoplasts were purified by floatation and cultured in liquid medium with glucose as described pre-

viously [8]. The hormones added were 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 μM α-naphtaleneacetic acid (NAA), and 2.2 μM benzyladenine (BA). The protoplasts were grown in 15 × 50 mm Costar Petri dishes at a density of 50–100 000 per ml. After 5 days the suspensions of protoplasts were diluted with culture medium. Ten to twelve days after protoplast isolation, small cell colonies were formed. The cultures were then diluted and agarose was added to give a final concentration of 0.2% as described [8]. After another 3–5 days the semi-solid agarose medium containing the minicalli was pipetted onto two different solid media. Medium 1 contained 90 mM sucrose and hormones as in the plating medium. Medium 2 contained 90 mM mannitol, 7 mM sucrose, 2.2 μM BA, and 0.5 μM NAA. Otherwise, medium 1 and 2 were identical [8]. After 7–14 days on the solid medium, calli were transferred to the regeneration medium to be tested. Twenty calli were placed in each 100 × 15 mm Petri dish. The standard regeneration medium was a modified MS medium with 30 mM sucrose, 21 mM NO_3^-, 8 mM NH_4^+, 4.6 μM zeatin and 0.6 μM IAA, other minerals and vitamins as specified for the TM4 medium of Shahin [12] (however, no choline chloride or MES was added). Standard growth conditions were 12 h cool-white light (Philips TL) 110 μE m^{-2} s^{-1} at 25 °C. After 6 weeks on the standard medium or the medium to be tested, the calli were transferred to another regeneration medium for elongation of shoot primordia (or formation of new shoots). This second medium was the same in all experiments, and the growth regulators of this medium were 4.4 μM BA and 0.3 μM gibberellic acid (GA_3), otherwise the medium was identical to the standard regeneration medium. After 4 weeks on the second regeneration medium, calli producing shoots were counted. Light conditions and temperature were the same for both regeneration media. Growth regulators and organic nitrogen compounds were added to part of the medium, pH was adjusted and the medium was sterilized by filtration and added to the rest of the medium after autoclaving.

When light quality was tested, the red light source was fluorescent lamps, Philips TL 20W red 15 with maximal emission at 660 nm. The blue light source was Philips TL 20W blue 18 with maximal emission at 450 nm. The white light source was Philips FT 20W white 32 de lux with maximal emission at 660 and 450 nm. The light intensity was regulated by several layers of white muslin. The standard incubation was 110 μE m^{-2} s^{-1} Philips TL 33 with maximal emission at 600 and 450 nm (cool-white). This cool-white light was also used when testing light intensity. Sixty calli were exposed to each treatment to be tested, and the experiments were repeated at least three times.

Results

No difference in regeneration was found between medium 1 and 2, and medium 1 was generally used for the callus-forming step.

Figure 1 shows the effect of various concentrations of zeatin and BA on the frequency of calli producing shoots. Zeatin at 4.6 and 9.2 μM gave the highest number of shoot-producing calli. Higher concentrations (23 and 46 μM) strongly inhibited growth and shoot induction. Benzyladenine was optimal at about 5 times higher concentrations than zeatin. Different concentrations of indoleacetic acid (IAA) (0, 0.06, 0.6, 6.0 μM) in combination with 4.4 μM BA were also tested, but no consistent effect of IAA was found (data not shown).

Various concentrations of sucrose and mannitol were examined (Fig. 2). Low sucrose concentrations favoured shoot induction. Sucrose at 3 mM, 15 mM, or 3 mM together with 27 or 87 mM mannitol gave the best results.

Shoot formation was induced at 0, 8 and 21 mM ammonium, but most efficiently at 8 mM. Nitrate concentrations at about twice the concentration of ammonium or higher were suitable for shoot formation (Fig. 3). The effect of some organic nitrogen compounds on differentiation was tested (Fig. 4). The addition of glutamine had no significant effect on shoot induction. Putrescine was slightly inhibitory on shoot induction at 0.5 mM and clearly inhibitory at 2 mM. Spermidine and spermine were strongly inhibitory on shoot induction as well as callus growth at 0.5 and 2 mM. Lower concentrations (0.5, 5, 50 μM) of the polyamines were also tested.

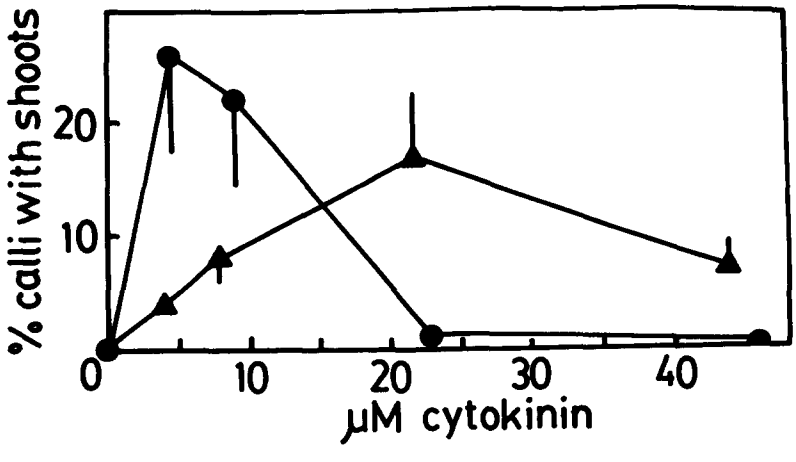

Fig. 1. The effect of various concentrations of zeatin (●) or BA (▲) on the frequency of calli producing shoots. Each point represents the mean of four independent experiments (240 calli). 1SE is indicated.

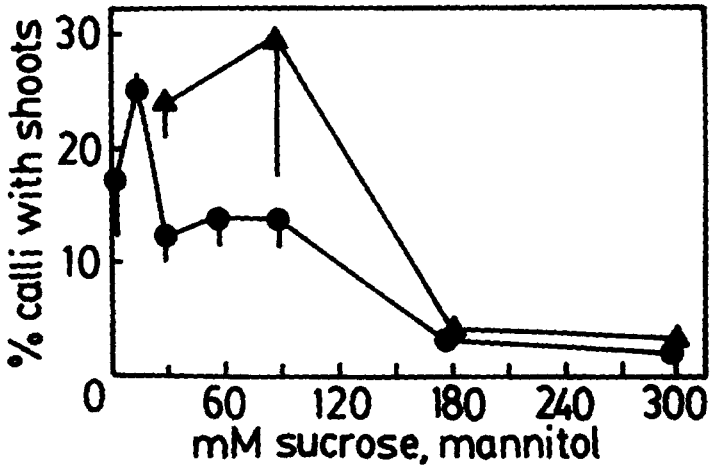

Fig. 2. The effect of various concentrations of sucrose (●) or mannitol in the presence of 3 mM sucrose (▲) on the frequency of calli producing shoots. Each point represents the mean of three different experiments (180 calli). 1SE is indicated.

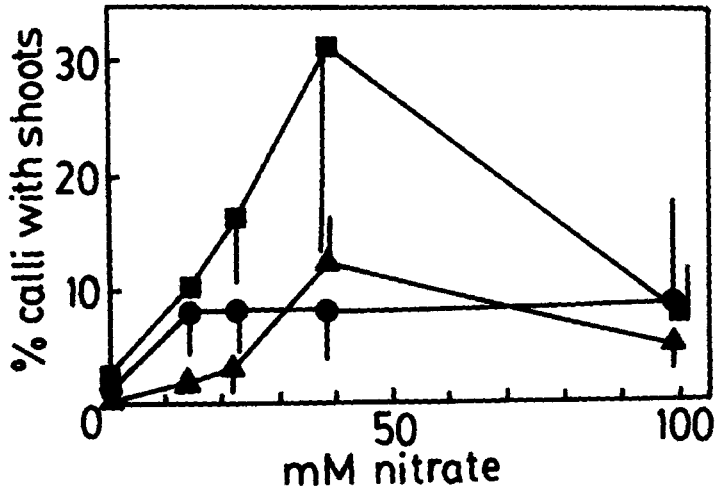

Fig. 3. The effect of various concentrations of nitrate at 0 (●), 8 (■) and 21 (▲) mM ammonium on the frequency of calli producing shoots. Each point represents the mean of three different experiments (180 calli). 1 SE is indicated.

Spermidine at 50 μM inhibited shoot formation, but otherwise no effects on shoot formation were observed at these lower concentrations.

Different light qualities and intensities were tested. Blue light or darkness inhibited shoot induction compared with white light. Red light was also inhibitory compared with white light. However, four times more calli

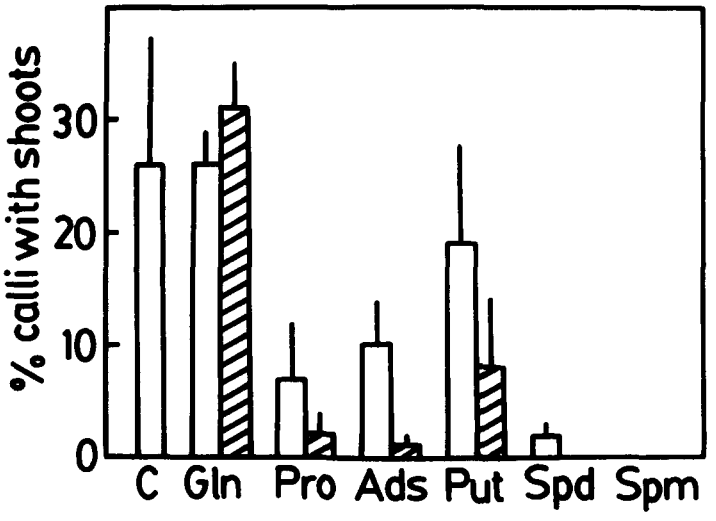

Fig. 4. The effect of various nitrogen compounds on the frequency of calli producing shoots. The compounds were tested at 0.5 mM (open bars) and 2 mM (hatched bars). The compounds tested were glutamine (Glu), proline (Pro), adenine sulphate (Ads), putrescine (Put), spermidine (Spd), and spermine (Spm). C represents the control with no additions. At 2 mM Spd and 0.5 and 2 Spm no shoots were formed. Each point represents the mean of three different experiments (180 calli). 1SE is indicated.

produced shoots in red light compared with blue light or darkness (Fig. 5A). High light intensity inhibited shoot induction compared to low light intensity. The experiments also indicated that 20 °C was more favourable for shoot induction than 25 °C (Fig. 5B).

Adjusted culture conditions were tested for five other genotypes. Table 1 shows the number of protoplast-derived calli producing shoots under standard conditions (first line) and at lower light intensity, temperature and sucrose concentrations. By adjusting the environmental conditions, increased shoot induction in all genotypes tested was obtained. The cultivar Beate produced fragile shoots, and very few shoots produced roots when transferred to hormone-free medium. All other genotypes tested rooted readily on hormone-free medium, and were successfully transferred to soil.

Discussion

The concentration of growth factors successfully used for regeneration of various cultivars of *S. tuberosum* by other investigators [3, 9, 13], i.e. 4.6 μM zeatin and 0.6 μM IAA, was well suited also for regeneration of the Rosa-

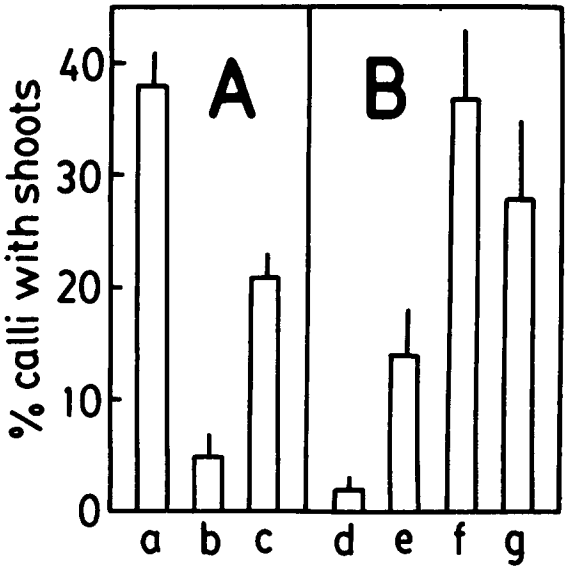

Fig. 5. The effect of (*A*) light quality, (*B*) light intensity and temperature on the frequency of calli producing shoots. The conditions tested were (a) warm-white, 5 μE m^{-2}s^{-1}, 24 h photoperiod, 25 °C; (b) blue, 5 μE m^{-2}s^{-1}, 24 h photoperiod, 25 °C; (c) red, 5 μE m^{-2}s^{-1}, 24 h photoperiod, 25 °C; (d) darkness, 25 °C; (e) cool-white, 110 μE m^{-2}s^{-1}, 12 h photoperiod, 25 °C; (f) cool-white, 30 μE m^{-2}s^{-1}, 12 h photoperiod, 25 °C; (g) cool-white, 110 μE m^{-2}s^{-1}, 12 h photoperiod, 20 °C. The figures represent the mean of 5 experiments (300 calli for each treatment). 1SE is indicated.

Table 1. The effects of light intensity, temperature and sucrose concentration on the regeneration of protoplast-derived calli in 6 different genotypes (100 calli per treatment).

Culture conditions			% calli with shoots					
Sucrose (mM)	Temp. (°C)	Light intensity (μE m^{-2}s^{-1})	× Rosamunda F × Aq	Beate	× Ruth	N73-20-262	N80-37-34	
30	25	110	14	19	0	2	29	28
30	25	30	37	40	1	0	38	53
15	25	30	–	53	1	2	–	43
30	20	30	–	60	24	28	–	–
15	20	30	–	68	25	30	–	–

munda breeding line tested here, and regeneration frequency could apparently not be easily improved by adjusting the concentrations of cytokinins or auxins.

Sucrose at concentrations of 60 to 90 mM is most commonly used in

regeneration media [10]. However, for protoplast-derived calli of potato, mannitol at 90 to 200 mM has frequently been used in combination with low (7.5 mM) sucrose concentration [5, 11, 13]. Our experiments also showed that low sucrose concentrations in combination with mannitol is suitable for regeneration in protoplast-derived calli of potato (Fig. 2). However, our experiments also showed that when the sucrose concentration was kept low, without mannitol, shoot formation was also efficiently induced. This indicates that high sucrose concentrations per se inhibit shoot induction in protoplast-derived calli. Similar results have been reported for protoplast-derived calli of *Nicotiana plumbaginifolia* [1] and *Brassica oleracea* [7].

The concentrations of nitrate and ammonium was not critical for good shoot induction as long as nitrate concentrations were kept at about twice the concentration of ammonium or higher. Thus, our standard conditions of 8 mM ammonium and 23 mM nitrate based on the protocol of Shepard [13], could apparently not be further improved.

Glutamine stimulates differentiation in several tissue culture systems [10] and has also been used in cultivation of *S. tuberosum* protoplast-derived calli [6]. However, in the present study no significant effect of glutamine was found. Adenine sulphate which is commonly added to culture media of protoplast-derived calli of *S. tuberosum* [5, 6, 11, 13] at concentrations of 0.2 to 0.4 mM reduced the number of calli producing shoots by about 70% at 0.5 mM. Lower concentrations were, however, not tested and could possibly be more beneficial.

Commonly, a light intensity of 80–100 $\mu E\,m^{-2}\,s^{-1}$ has been used in regeneration of protoplast-derived calli of *Solanum* [3, 5, 13]. In our experiments with the Rosamunda breeding line, reducing the light intensity was an important adjustment for efficient shoot induction (Fig. 5). In further experiments with three other cultivars and breeding lines the importance of low light intensity (30 $\mu E\,m^{-2}\,s^{-1}$) was confirmed, but for another two genotypes lowering the temperature from 25 to 20 °C was also necessary to achieve regeneration (Table 1). These experiments show that for various genotypes of *S. tuberosum* low light intensity and temperature are essential for good shoot regeneration.

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