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# The effect of antibiotics on the inhibition of callus induction and plant regeneration from cotyledons of sugarbeet (*Beta vulgaris* L.)

**Dave W. Catlin**

Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568, USA

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## Abstract

Callus induction and plantlet regeneration from cotyledonary explants of sugarbeet was observed utilizing two media formulations, MS and a modified MS termed RVIM both supplemented with 1.0 µg/ml BAP as the sole growth regulator. Callus induction was genotype dependent. The USDA line 8787 produced the highest response for callus induction followed by 'Betaseed 4587' and the USDA line C600. This order was conserved on both media formulations. Shoot induction was consistently higher averaging 32% from the RVIM formulation over the 3 genotypes compared to 25% from MS. The antibiotics geneticin, gentamycin, hygromycin, kanamycin and phleomycin were screened with the modified RV system utilizing 'Betaseed 4587'. Callus growth was inhibited by levels of 50 µg/ml geneticin, 150 µg/ml gentamycin, 10 µg/ml hygromycin, 150 µg/ml kanamycin and 20 µg/ml phleomycin. The results indicate that the concentrations of antibiotics used to inhibit callus induction will be sufficient for use as selectable markers in transformation experiments with *Beta vulgaris*.

## Abbreviations

$B_5$ basal medium (Gamborg *et al*, 1968)
BAP, $N^6$-Benzylaminopurine
IBA, Indole-3-butanoic acid
MS basal medium (Murashige and Skoog 1968)
RVIM, modified MS basal medium (Freytag *et al*, 1988)
MES, (2[N-Morpholino] ethanesulfonic acid

## Introduction

Sugarbeet (*Beta vulgaris* L.) is an important member of the family Chenopodiaceae. In an effort to aid in the improvement of its culture, protocols and methodologies for its *in vitro* manipulation are desirable.

During the past few years, progress has been made in developing protocols for the regeneration from various sugarbeet explant sources including hypocotyls (Van Geyt and Jacobs 1985; Saunders and Shin 1986), petioles (Saunders and Doley 1986; Saunders and Shin 1986), leaves (Doley and Saunders 1989; Mikami *et al.* 1989; Yu 1989), ovules (Goska and Jassem 1988; Doctrinal 1989) and protoplasts (Krens and Jamar 1987). In only two cases have there been reports, that the author is aware, of *in vitro* regeneration *via* cotyledonary explants, (Butenko *et al.* 1972; Tetu *et al.* 1987), the latter reporting unsuccessful attempts.

There have been no reports on the effects of antibiotics used as selectable markers on *in vitro* regeneration systems in sugarbeet. It was the aim of this study to first develop a reliable protocol for the regeneration *via* cotyledonary explants and to screen for levels of callus inhibition using several antibiotics. This report describes a reliable and reproducible method for callus induction and plant regeneration from cotyledonary explants and the effects of several antibiotics on the inhibition of this process.

## Materials and Methods

### *A. Callus induction and plant regeneration*

Seed from a commercial hybrid, 'Betaseed 4587' (Shakopee, MN), and two annual breeding lines, C600 and 8787, (R. Lewellen, USDA, ARS, Salinas, CA), were surface disinfected by submersion in distilled water

at 55°C for 15 minutes followed by 95% ethanol for 1 minute and finally a 5 minute soak in a 20% household bleach solution. Subsequently the seeds were rinsed in 3x double-distilled sterile water. Seeds were germinated on 1.5% water agar. Incubation was at 24°C, 10 $\mu E/m^{-2}/s^{-1}$. After 9 days the cotyledons were aseptically excised and the proximal half was plated directly to regeneration medium consisting of either MS (Murashige and Skoog 1962) or RV solidifed with 0.25% Gelrite, pH 5.7. Each medium was supplemented with 1.0 µg/ml BAP and 2.5% sucrose. In the case of MS, standard $B_5$ vitamins (Gamborg *et al.* 1968) were added and in the RVIM medium, (RV from Freytag *et al.* 1988). This medium was amended with inositol, 100 µg/ml and MES, 100 µg/ml) together with 10 vitamins and 6 amino acids from Freytag *et al.* The cultures were maintained at 24°C, 10 $\mu E/m^{-2}/^{-1}$, with a 16-h photoperiod. After shoots developed from surrounding callus they were removed and transferred to a conditioning medium consisting of 1/4 MS basal salts, 1% sucrose solidified with 0.8% Difco purified agar. When shoots were 2-3 cm in length, they were transferred to a rooting medium consisting of $B_5$ basal salts, 1% sucrose, 3.0 µg/ml IBA, 200 µg/ml activated charcoal solidified with 0.8% Difco bacto agar in 100x25 mm Kimax test tubes. The rooted plantlets were incubated *in vitro* at 4-5°C for 3 months to fulfill the vernalization requirements for bolting and seed set. Observations for callus and shoot induction were made after 4 weeks then weekly for an additional 4 weeks. Each experiment was duplicated 4 times with 30-40 replicates per treatment.

*B. Effects of antibiotics on callus induction and regeneration*

The antibiotics used in this study; geneticin, gentamycin (Sigma Chem Co), hygromycin, kanamycin (Boehringer Mannheim Co.) and phleomycin (Cayla S.A.R.L., Toulouse, France), were all filter sterilized and added to the media after autoclaving. Cotyledonary explants of 'Betaseed 4587' were cultivated in RVIM media supplemented with the antibiotics geneticin, gentamycin, hygromycin, kanamycin or phleomycin (Table 2). Observations were made after 4 weeks and then weekly for the next 4 weeks. Each experiment was repeated at least once with 30-40 replicates per treatment. Quantification of callus induction was based on either the presence or absence of callus after 8 weeks. Shoot induction was quantified by adding the total number of shoots per explant with callus after 8 weeks. Analysis of variance was performed using the least significant difference for both number of explants forming calli and number of shoots.

## Results and Discussion

### *A. Callus induction and plant regeneration*

The frequencies of callus and shoot induction for each of the 3 genotypes tested are listed in Table 1.

**Table 1.** Mean frequencies of cotyledonary callus induction and shoot formation by three sugarbeet lines on two media formulations.

| Lines | Media type | Frequency (%) Callus | Shoots[1] |
|---|---|---|---|
| 'Betaseed 4587' | RVIM | 84 | 33 |
| 'Betaseed 4587' | MS | 73 | 30 |
| USDA C600 | RVIM | 46 | 32 |
| USDA C600 | MS | 50 | 30 |
| USDA 8787 | RVIM | 100 | 30 |
| USDA 8787 | MS | 100 | 14 |

[1] Expressed as a % of shoots from explants forming callus

Based on these results for callus induction with 'Betaseed 4587' and C600 for the two media formulations tested, RVIM and MS, the additional amino acids and vitamins of the former did not result in a significantly higher callus or shoot induction (t=0.98, 6df, p level 0.01). On the other hand, significant differences for callus induction were observed among the three different sugarbeet lines indicating genotypic differences (t=10.2, 2df, p level 0.01). Shoot induction was consistently higher with all lines when the RVIM formulation was used. Although 100% of the explants in the 8787 genotype produced callus, much of it was hard, nodular, and non-regenerative. Only callus that was friable and cream colored was capable of differentiating into shoot meristems (Figure 1). In most cases callus developed in 3-4 weeks, shoots in 4-6 weeks, and roots in 1-2 weeks (Figure 2). The remainder of the experiments described here utilize the sugarbeet line 4587 on RVIM medium, and this determination is based on a consistently higher level of shoot induction for the genotype 4587.

**Fig. 1.** Shoot induction from callus of cotyledon explant on RVIM medium supplemented with 1.0 µg/m BAP.

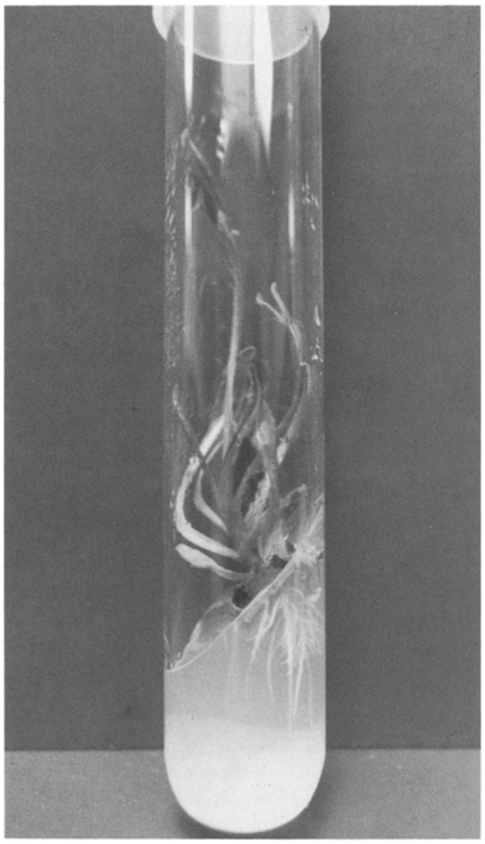

**Fig. 2.** Root induction of sugarbeet plantlet on $B_5$ medium supplemented with 3.0 µg/m IBA.

### *B. Effects of antibiotics on callus induction and regeneration*

Based on the results obtained in part A, the next step was to evaluate the response of various antibiotics to the inhibition of callus growth and shoot development.

**Table 2.** Effect of antibiotics on callus and shoot induction from cotyledonary explants of 'Betaseed 4587'.

| Antibiotics | (µg/ml) | Explants forming calli No. | (%) [1] | Shoots No. | (%) [1] | Avg. number of shoots per explant with callus |
|---|---|---|---|---|---|---|
| Kanamycin | 0 | 97[a] | (100) | 103[b] | (100) | 1.0 |
| | 10 | 103[a] | (106) | 215[a] | (209) | 2.0 |
| | 20 | 109[a] | (112) | 214[a] | (208) | 1.0 |
| | 30 | 199[a] | (102) | 180[a] | (175) | 1.4 |
| | 40 | 89[a] | (92) | 52[c] | (50) | 0.4 |
| | 50 | 94[a] | (97) | 15[c] | (14) | 0.2 |
| | 75 | 81[a] | (83) | 5[c] | (6) | 0.03 |
| | 150 | 3[b] | (3) | 0[d] | (0) | 0 |
| Gentamycin | 0 | 100[a] | (100) | 73[a] | (100) | 0.7 |
| | 25 | 97[a] | (97) | 78[a] | (107) | 0.7 |
| | 50 | 93[a] | (93) | 64[a] | (87) | 0.6 |
| | 75 | 92[a] | (92) | 50[a] | (68) | 0.4 |
| | 100 | 79[b] | (79) | 12[b] | (16) | 0 |
| | 125 | 18[b] | (18) | 0[c] | (0) | 0 |
| | 150 | 6[c] | (6) | 0[c] | (0) | 0 |
| Geneticin | 0 | 69[a] | (100) | 68[a] | (100) | 0.7 |
| | 10 | 53[a] | (77) | 0[b] | (0) | 0 |
| | 25 | 5[b] | (7) | 0[b] | (0) | 0 |
| | 50 | 0[b] | (7) | 0[b] | (0) | 0 |
| | 75 | 0[b] | (0) | 0[b] | (0) | 0 |
| | 100 | 0[b] | (0) | 0[b] | (0) | 0 |
| Hygromycin B | 0 | 86[a] | (100) | 77[a] | (100) | 0.7 |
| | 10 | 0[b] | (0) | 0[b] | (0) | 0 |
| | 15 | 0[b] | (0) | 0[b] | (0) | 0 |
| | 20 | 0[b] | (0) | 0[b] | (0) | 0 |
| | 25 | 0[b] | (0) | 0[b] | (0) | 0 |
| Phleomycin | 0 | 92[a] | (100) | 87[b] | (100) | 0.8 |
| | 1 | 92[a] | (100) | 141[a] | (162) | 0.4 |
| | 5 | 73[a] | (79) | 55[b] | (63) | 0.05 |
| | 10 | 42[b] | (46) | 0[c] | (0) | 0 |
| | 15 | 19[b] | (21) | 0[c] | (0) | 0 |
| | 20 | 0[c] | (0) | 0[c] | (0) | 0 |

[1] Expressed as a percent of controls

For callus induction, the treatment effect produced statistically significant differences compared to the control at the 0.05 level at 150 µg/ml kanamycin, 100 µg/ml gentamycin, 50 µg/ml geneticin, 5 µg/ml hygromycin and 5 µg/ml phleomycin. The lower treatment levels for each antibiotic were not significantly different from the control. For shoot induction, two observations were made based on analysis of variance at the 0.05 level. Kanamycin at 10-30 µg/ml and phleomycin at 1.0 µg/ml resulted in significantly higher shoot induction, whereas levels above 40 µg/ml kanamycin, 125 µg/ml gentamycin, 10 µg/ml geneticin, 5 µg/ml hygromycin and 5 µg/ml phleomycin were all significantly inhibitory to shoot induction.

Several authors have reported enhanced regeneration when antibiotics were included in the media formulation. M.L. Roberts *et al.* (1989) reported the growth promoting activity of certain beta lactam antibiotics on the growth of *Bouvardia ternifolia* calli and that it appeared to be dependent on the presence of an auxin component in the penicillin molecule, but no promotive response was seen with kanamycin. Mathias and Makusa (1987) reported a stimulatory effect of cefotaxime on growth and regeneration of *Hordeum vulgare* L. callus. They postulated that the observed enhanced activity could be a result of cefotaxime mimicking a plant growth regulator, although no data was provided to substantiate this. It is possible that one or more of these actions could be responsible for the promotive effect seen here for shoot induction from callus or it may be possible that a breakdown product of the aminoglycoside could have growth promoting activity at low concentrations.

It can be seen that while all of the antibiotics tested are inhibitory to callus and shoot induction, the dose reponse is quite different. The first group comprising kanamycin, gentamycin, geneticin, hygromycin and phleomycin all

act by inhibition of ribosomal protein synthesis whereas phleomycin works by DNA cleavage. This differential response may be due to different affinities for binding sites to inhibit protein synthesis and perhaps differences in the permeabilities based on structural activity relationships along with different modes of action. Based on these results, the effectiveness of each antibiotic for callus and shoot inhibition in descending order was hygromycin > geneticin > phleomycin > kanamycin > gentamycin.

When using antibiotics as selectable markers during transformation experiments, questions should be addressed as to how much and when the selection pressure should be applied. Whether to select at low antibiotic levels possibly allowing a high degree of escapes, or to select at high levels early on then remove the cells from the presence of the antibiotic, or still to select at low levels and gradually increase the antibiotic concentration before regeneration is attempted.

In conclusion, a system for the regeneration of sugarbeet *via* cotyledonary explants has been tentatively developed together with screening protocols for callus and shoot inhibition using several antibiotics. This should prove useful for the development of methodologies for the selection of transformed callus within *Beta vulgaris* L.

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