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In vitro shoot regeneration from leaf discs of *Betula pendula* 'Dalecarlica' EM 85

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Abstract

The effect of zeatin, NAA (α-naphthaleneacetic acid), putrescine and cefotaxime on the frequency of shoot regeneration from *Betula pendula* 'Dalecarlica' EM 85 leaf discs has been examined. About 80% of leaf discs were induced to form adventitious shoots when the culture medium contained 45.6 μmol l^{-1} zeatin and 0.1 mmol l^{-1} cefotaxime. The addition of NAA to zeatin-containing media prevented shoot regeneration but stimulated root development directly from leaf tissues. Putrescine (0.1 mmol l^{-1}) and cefotaxime (0.1 mmol l^{-1}) could both significantly increase the percentage of leaf discs regenerating on optimal zeatin-containing media, and increase the number of shoots per regenerating disc.

Abbreviations: NAA — α-naphthaleneacetic acid, BAP — 6-benzyladenine

Introduction

High-frequency shoot regeneration from adult material is an essential and often critical step in many programmes of genetic improvement by somatic methods, particularly in woody species, where regeneration is often regarded as difficult [6, 7]. Leaf discs from a micropropagation system have been previously shown to be a suitable material for this purpose [1].

Several authors have reported the in vitro culture of *Betula pendula* to be a suitable system both for clonal propagation of good genotypes [3, 4, 10] and for physiological studies [9].

The clone 'Dalecarlica' EM 85 is currently in short supply both commercially and as a research tool (T. Marks, pers. comm.). As birch plants regenerated from callus [9], leaf and roots seem to be genetically stable [10], in vitro regeneration could also represent a technique to improve mass propagation of this clone.

The aim of this work was to evaluate the use of leaf discs as source material for regeneration studies and to test the effects of various organic additions to the medium. Zeatin has already been shown to be effective in inducing regeneration in birch [10] whilst putrescine and cefotaxime can aid regeneration from leaf discs in other tree species such as apple [1] and pear [13].

Materials and methods

Shoot cultures of *Betula pendula* 'Dalecarlica' EM 85 were micropropagated on WPM (Woody Plant Medium) [2] with 2.5 μmol l^{-1} BAP.

Leaves from proliferating shoots were aseptically collected from the length of the stems and 7 mm diameter leaf discs were excised aseptically using a cork borer. The discs were cultured in multiwell replidishes (Sterilin, UK), one disc per well, with their upper side in contact with the medium, which

contained 163 μmol l^{-1} adenine and varying concentrations of zeatin, NAA, putrescine and cefotaxime.

In a preliminary trial, four zeatin levels (0, 11.1, 22.8 and 45.6 μmol l^{-1}) were tested either alone or in combination with NAA (0, 1.3, 2.6, 5.3 and 10.7 μmol l^{-1}) or putrescine (0, 0.01, 0.1, 1 and 10 mmol l^{-1}) both in dark and light growth conditions (approx. 1000 lux, photoperiod 16 h light/8 h dark), at 25°C. Five replicate leaf discs were used for each treatment and growth condition.

Since NAA failed to induce regeneration in combination with zeatin the auxin was omitted in subsequent work and only the highest concentration of zeatin (46.5 μmol l^{-1}) used in combination with three putrescine (0, 0.1, and 1.0 mmol l^{-1}) and four cefotaxime concentrations (0, 0.1, 0.2 and 0.4 mmol l^{-1}). Each of these two substances were tested with the highest zeatin concentration but not with each other.

Cefotaxime (mol. wt. = 477) was used as 'Claforan' (Roussel Laboratories Ltd.), a cephalosporin antibiotic containing approximately 4.8% of sodium. Both cefotaxime and putrescine were filter-sterilized (Millipore, 0.02 μm) and added aseptically after filter sterilization to the freshly autoclaved medium.

Data were recorded weekly and the discs that showed adventitious shoot formation were transferred to fresh medium containing 2.5 μmol l^{-1} BAP.

Statistical treatments

The percentage of leaf discs that regenerated adventitious shoots was recorded. Percentage differences were examined using the normal approximation to a binomial distribution. The effect of zeatin concentration on the percentage of regenerating leaf discs was calculated using a regression analysis.

Results

After two weeks all the discs cultured at each NAA level, regardless of zeatin concentration and growth conditions, produced roots rising from the callus that grew along the cut edge of the discs. No adventitious shoots were formed in the presence of the auxin. Both callus and roots appeared white in the dark but showed red pigmentation in the light. In the dark, moreover, the roots did not show a positive geotropic response. In the absence of auxin, sporadic root formation was observed only at the three highest putrescine levels.

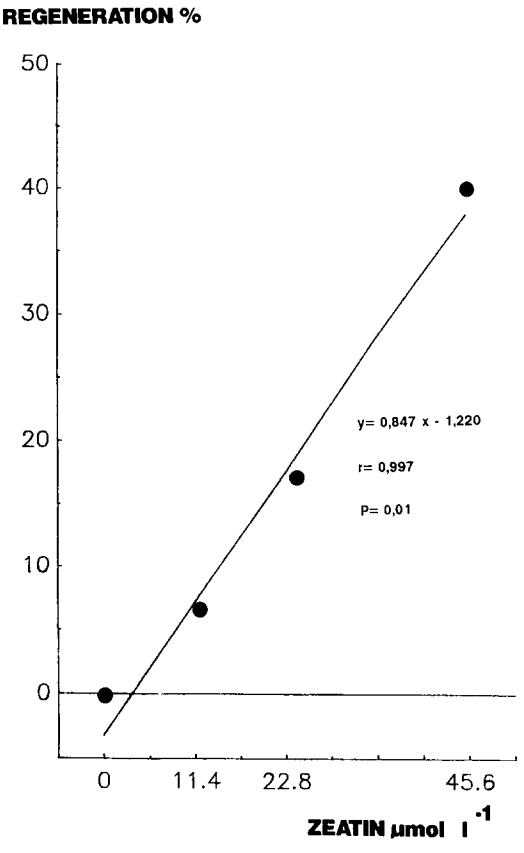

Fig. 1. Correlation between the zeatin level and the percentage of regenerating leaf discs. Data collected on total 125 replicates, and 25 replicates per zeatin concentration. Cefotaxime and putrescine not present.

Shoot regeneration was obtained only in the light and only if zeatin was present in the culture medium. A positive linear regression between the cytokinin level and the percentage of regenerating discs was also found (Fig. 1). Leaf discs cultured on 0.1 mmol l^{-1} cefotaxime and 1 mmol l^{-1} putrescine with the highest level of zeatin, regenerated shoots after two weeks but the highest regeneration percentages were recorded in all cases after four weeks.

The adventitious shoots arose mostly from the callus formed at the disc edge and in particular from the disc side in contact with the medium. The number of regenerating shoots per disc that were

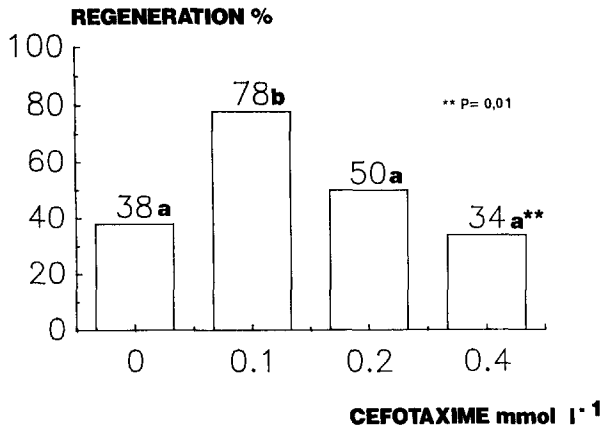

Fig. 2. The effect of cefotaxime on the percentage of regenerating leaf discs. Data collected on a total of 200 leaf discs (replicates) cultured on 45.6 μmol l^{-1} of zeatin in the absence of putrescine. (Values with different suffix differ significantly at $^{**}P = 0.01$, $^{*}P = 0.05$.)

countable in the first three weeks of growth never exceeded two or three. After this period of time it was not possible to tell if further shoots were developing de novo or from adventitious shoots already laid down. There were no consistent effects of putrescine on shoot number per disc at any of the five concentrations added except that at 10 mmol l^{-1} some inhibition was evident (data not shown). The effects of cefotaxime on shoot number per disc were not assessed.

Cefotaxime at 0.1 mmol l^{-1} added to the highest zeatin concentration (45.6 μmol l^{-1}) increased the percentage of discs regenerating adventitious shoots from 38 to 78% after four weeks in culture (Fig. 2). Higher concentrations of cefotaxime progressively reduced this promoting effect. Putrescine at 1 mmol l^{-1} in combination with the optimal zeatin concentration was partly able to substitute for cefotaxime but only induced 58% of the leaf discs to undergo organogenesis. A lower concentration of putrescine, 0.1 mmol l^{-1}, induced 50% of the discs to undergo regeneration, a significant increase over the control value of 38% (data not shown).

Discussion and conclusions

From the present work, zeatin, together with light, seemed to be the key factors for shoot regeneration from leaf discs of *Betula pendula* 'Dalecarlica' EM 85. This was obtained by using zeatin in WPM plus adenine, and using light growth conditions.

The presence of NAA in a zeatin-containing medium was reported to allow shoot regeneration from leaf callus of *Betula pendula* F. purpurea [3] while in our conditions, it was shown to inhibit shoot production and stimulate root formation. As previously reported [4], these roots were very pubescent and lacked a positive geotropic response in the dark.

A low level of putrescine enhanced regeneration, probably because of its stimulating effect on cell division as observed in mesophyll protoplasts of *Alnus glutinosa* [5]. As bud initiation in leaf disc cultures of *Passiflora* was accompanied by the highest endogenous putrescine content, Harsha & Metha [11] suggested that putrescine played a role in bud induction. Moreover, this polyamine has been shown to enhance regeneration in apple leaf discs [12] when used at the concentrations which have also been effective in *Betula* regeneration.

The highest regeneration percentage was obtained with cefotaxime, which is currently and successfully used for the in vitro regeneration of wheat embryos and some fruit trees, e.g. pear [13] and apple [14]. Mathias & Boyd [8] found that this antibiotic stimulated shoot regeneration from wheat calli initiated and maintained on 60 μg ml^{-1} of cefotaxime. Mathias & Mukasa [15] subsequently showed similar stimulatory effects on the growth and regeneration of four varieties of barley. They ruled out any indirect effects due to a suppression of endogenous bacterial infection since they had never observed any systemic infections of their tissue cultures, originally derived from immature embryos. Whilst this is a reasonable assumption for material with a seed origin the same is not true of vegetative tissues which are introduced into culture. The latter will at one time have been exposed to 'field conditions' and consequently microbial attack. It is not possible therefore to rule out the possibility that its effects may be indirect in the case of birch, possibly by its ability to remove or reduce toxic microbial compounds from the medium or by reducing competition for nutrients. This would be in addition to its other postulated role as a plant growth regulator in its own right [8]. Both indirect and direct effects may be responsible for the stimulation of regeneration in birth.

Betula pendula has been shown to regenerate

adventitious shoots from the base of in vitro cultured buds [9], from callus [3], and root and leaf cultures [10]. The leaves used to regenerate shoots of *Betula pendula* Roth. [10] were distinct in origin compared to those used in the present study in that they were larger and were taken from the base of the plantlets. In some cases where these leaves were in contact with the medium they also showed signs of meristematic activity as evidenced by callus formation. The significance of this in subsequent regeneration experiments was not commented upon by the authors.

In our conditions, working with the clone 'Dalecarlica', we have been able to initiate a simple and efficient method for regenerating shoots using leaves from in vitro shoot cultures.

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