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Regeneration of shoots from embryo hypocotyls of common ash (*Fraxinus excelsior*)

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Summary

The addition of thidiazuron (TDZ) to MS salts and vitamins, instead of benzylaminopurine (BAP) increased both the culture weight and the proportion of ash embryo hypocotyl explants that produced adventitious shoots. The concentration of BAP, but not that of TDZ also affected these parameters. Addition of 1-naphthalene acetic acid reduced culture performance, indolebutyric acid (IBA) was beneficial, and 2,4-dichlorophenoxyacetic acid had no effect. During both 1990 and 1991, rates of regeneration declined as the growth season progressed. Adventitious shoots, which were also obtained from hypocotyls from dried seeds, were established as proliferating shoot cultures following transfer to DKW medium with 5.0 mg l^{-1} BAP, and the resulting shoots were rooted in half-strength Woody Plant medium with 1.0 mg l^{-1} IBA.

Introduction

Many *in vitro* techniques are available for the genetic manipulation of plants leading to the generation of somaclonal variants, haploids and dihaploids, somatic hybrids and transgenic plants. Almost all of these *in vitro* procedures are dependent upon reliable methods to regenerate plants from cells or tissues. Thus far, most progress has been achieved with species of Solanaceae and Cruciferae for which techniques for adventitious regeneration are now well established. Attention has focussed recently upon the potential applications of non-conventional genetic manipulation to trees. There has been success in this respect with certain temperate fruit trees, and some progress has also been achieved with forest species (Hammatt 1992).

Common ash (*Fraxinus excelsior*) is an important temperate broadleaved tree found widespread throughout the British Isles and Northern Europe. It is used principally for furniture and there are several ornamental forms. There are only a few reports on the tissue culture of *Fraxinus* species. Successful micropropagation, by axillary shoot proliferation, has been reported recently for *F. americana* (white ash; Preece *et al.* 1987) and *F. excelsior* (Chalupa 1990; Hammatt and Ridout 1992). Adventitious regeneration in the genus has been achieved, thus far, only through the formation of somatic embryos from embryo cotyledons of *F. americana* (Preece *et al.* 1989). The present study investigated the response of common ash tissues *in vitro* to various growth regulators at a range of concentrations in order to identify optimum conditions for adventitious regeneration of shoots from embryo hypocotyls.

Materials and Methods

Plant material

Fresh seeds of *F. excelsior* were obtained from a 70 year-old tree, accession number 2228, growing at Horticulture Research International, East Malling. Dried seeds of British origin, were obtained from Forestart, Shrewsbury, UK. Seeds which were infested with the caterpillar of the moth *Pseudargyrotoza conwagana*, often had small holes through their testas, and were discarded. All seeds were surface-sterilised using 12% (v/v) Domestos commercial bleach solution [0.96% (v/v) NaOCl; Lever Bros., UK] for 10 min., followed by 5-6 rinses in sterile water. Dried seeds were soaked, for 48 h at 4°C, in sterile water, and then immersed in 12% (v/v) Domestos for a further 10 min., followed by thorough rinsing. Dried seeds that retained their dark brown colour, after both sterilisation treatments, had usually been damaged by *P. conwagana*, and were discarded. The testas were slit laterally and the

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two halves were teased apart to expose the embryo within. As required, hypocotyls were isolated by cutting 2 mm below the cotyledonary nodes.

Each isolated embryo hypocotyl was transferred to 8 ml of autoclaved, agar-solidified regeneration medium in a Coulter Counter cup. Shoot cultures were established in glass honey jars (Astell Scientific, Sidcup, UK) containing 50ml of culture medium. Shoots were rooted in Coulter Counter cups.

Culture media

The hormone-free salt and vitamin mixture of Murashige and Skoog (1962) (MS) was used for regeneration experiments, that of Driver and Kuniyuki (1984) (DKW) was used for micropropagation of regenerated shoots, and half-strength Woody Plant medium (Lloyd and McCown, 1980) was used for rooting. These media were supplemented sometimes with 0.1, 1.0 or 5.0 mg l^{-1} of N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron; TDZ) or benzylaminopurine (BAP) and 0.1mg l^{-1} of 1-naphthaleneacetic acid (NAA), indole butyric acid (IBA) or 2,4-dichlorophenoxyacetic acid (2,4-D). All media were solidified with 6.0 g l^{-1} Sigma agar, and adjusted to pH 5.7 prior to sterilisation (121°C, 15 min).

Arrangement of cultures

All cultures were arranged as randomized block designs in a controlled environment at 24°C with a 16h daily photoperiod of 0.9-1.0 m mol m^{-2} s^{-1}, provided by Philips 125W white fluorescent tubes, positioned 24 cm overhead.

Data analysis

Unless stated otherwise, all means were calculated from 24 explants per treatment four weeks after establishment of the cultures. Logistic regression methods (Cox & Snell, 1989) were used to analyze proportions. This approach, whilst broadly analogous to analysis of variance, makes proper allowance for the binary nature of these response variables. Culture masses were transformed to $\log_{10}$ values before analysis of variance.

For single percentage values in Fig. 1, 95% confidence limits were calculated using the method of Fujino (1980).

Results

Effects of cytokinins, auxins and sampling dates on regeneration from embryo hypocotyls from fresh seeds

Four replicate experiments were carried out in 1990, at 14 day intervals on 24/8, 7/9, 21/9 and 5/10, in order to examine the effects of different growth regulators upon regeneration. MS medium was supplemented with TDZ or BAP, each at one of four concentrations (0, 0.1, 1.0 and 5.0 mg l^{-1}), either without auxin or with NAA, IBA or 2,4-D at 0.1 mg l^{-1}, resulting in 28 different combinations of growth regulators.

Five days after initiation of the cultures, most of the embryo hypocotyl explants had turned green. After seven

Table 1. Effect of different concentrations of benzylaminopurine and thidiazuron upon the percentage of cultures that produced adventitious shoots or died, and upon culture weights.

	Percentage of explants that produced adventitious shoots		Percentage of explants that died		Log_{10} culture fresh weight (g)	
	Cytokinin used					
Concentration	BAP	TDZ	BAP	TDZ	BAP	TDZ
None	8		38		1.14	
0.1	41	70	28	24	1.5	1.98
1.0	59	73	37	25	1.56	2.04
5.0	65	77	27	23	1.76	2.08
LSD 5%	5.2		5.3		0.13	
(81 d.f.) 1%	9.2		9.2		0.17	
0.1%	11.9		11.9		0.21	

Means were averaged over all auxin treatments. Twenty explants were used per medium in each of four replicate experiments.

days, the first signs of regeneration were detected as small green protuberances from the cut end of the hypocotyl and from those areas of the explant that had been damaged during extraction from the seed. Within three weeks, leaves were observed, and after a month, distinct shoots were produced.

Effects of cytokinins on regeneration

While shoots were obtained from 8% of explants on culture medium lacking cytokinin, the inclusion of BAP or TDZ significantly increased adventitious shoot formation ($p<0.001$) and culture fresh weights ($p<0.001$), and reduced culture deaths ($p<0.01$) (Table 1). Regeneration was more common ($p<0.001$) and fresh weights were greater ($p<0.001$) with TDZ than with BAP at all concentrations tested. While the proportions of explants that died were lower with TDZ than with BAP, this was only significant at 1.0 mg l^{-1}. Culture fresh weights tended to increase with TDZ concentration, though the differences between means were not significant. Regeneration increased significantly with TDZ concentration ($p<0.05$ for the difference between 0.1 and 5.0 mgl^{-1}) and also with concentration of BAP, while fresh weight was greater with BAP at 5.0 mg l^{-1} than with either 0.1 ($p<0.001$) or 1.0 ($p<0.01$) mg l^{-1}.

Effects of auxins on regeneration

None of the auxins used significantly affected explant deaths or regeneration (Table 2). Significant differences were observed with culture mass, which was reduced by NAA ($p<0.001$) and increased by IBA ($p<0.001$).

Effects of sampling date on regeneration

When results from all of the culture media used on each date in 1990 were combined, the rates of regeneration decreased and the proportion of necrotic cultures increased as the growth season progressed (Fig. 1). Rates of regeneration from embryo hypocotyl explants, cultured at two weekly intervals during the 1991 growth season, on MS medium with 0.1 mg l^{-1} each of TDZ and IBA, also decreased significantly after 11/10, but rates of death were not affected.

Effect of thidiazuron concentration on hypocotyl explants from dried seeds

Embryo hypocotyls from dried seeds were incubated on MS medium with 0, 0.001, 0.01, 0.1, 1.0 and 5.0 mg.l^{-1} TDZ. Combined results from three replicate experiments showed a strong effect of TDZ concentration ($p<0.001$) on regeneration (Table 3), rates of shoot formation being greatest at the three highest concentrations. Cultures grown with TDZ sometimes produced vitrified shoots, vitrification increasing with TDZ concentration ($p<0.001$). Of the TDZ levels tested, 0.1 mg l^{-1} gave excellent rates of regeneration and acceptable levels of vitrification.

Table 2. Effect of different auxins upon the percentage of cultures that produced adventitious shoots or died, and upon culture weights.

Auxin (0.1 mgl^{-1})		Percentage of explants that produced adventitious shoots	Percentage of explants that died	Log_{10} culture fresh weight (g)
None		57	27	1.73
NAA		48	32	1.55
2,4-D		58	31	1.72
IBA		61	25	1.90
LSD	5%	11.9	10.7	0.10
(81 d.f)	1%	15.8	14.2	0.1
	0.1%	20.3	18.3	0.17

Means were averaged over all cytokinin treatments. Twenty explants were used per medium in each of four replicate experiments.

Table 3. Effect of TDZ concentration upon the mean proportion (±s.e.) of ash hypocotyl explants, extracted from dried seeds, that produced shoots and upon the proportion that produced vitrified shoots.

Concentration of TDZ (mg l^{-1})		Percentage of explants that produced adventitious shoots	Percentage of explants that produced vitrified adventitious shoots
0		3.0±2.0	0
0.001		1.0±1.0	0
0.01		53±5.9	1.0±1.3
0.1		96±2.4	10±3.4
1.0		89±3.8	24±5.0
5.0		92±3.2	33±5.3
LSD	5%	11.1	13.2
(18 d.f.)	1%	15.5	18.5
	0.1%	22.5	26.6

Fig. 1. The percentage of ash embryo hypocotyl explants from fresh seeds (with 95% confidence limits), that either died (- - ■ - -) or produced adventitious shoots (——▶——) on different dates during the 1990 and 1991 growing seasons. Data for 1990 are the means of 28 different culture media and for 1991 the percentages were obtained with only one medium, specifically MS with 0.1 mg l^{-1} each of TDZ and IBA. Differences in sample size explain the larger confidence intervals for 1991.

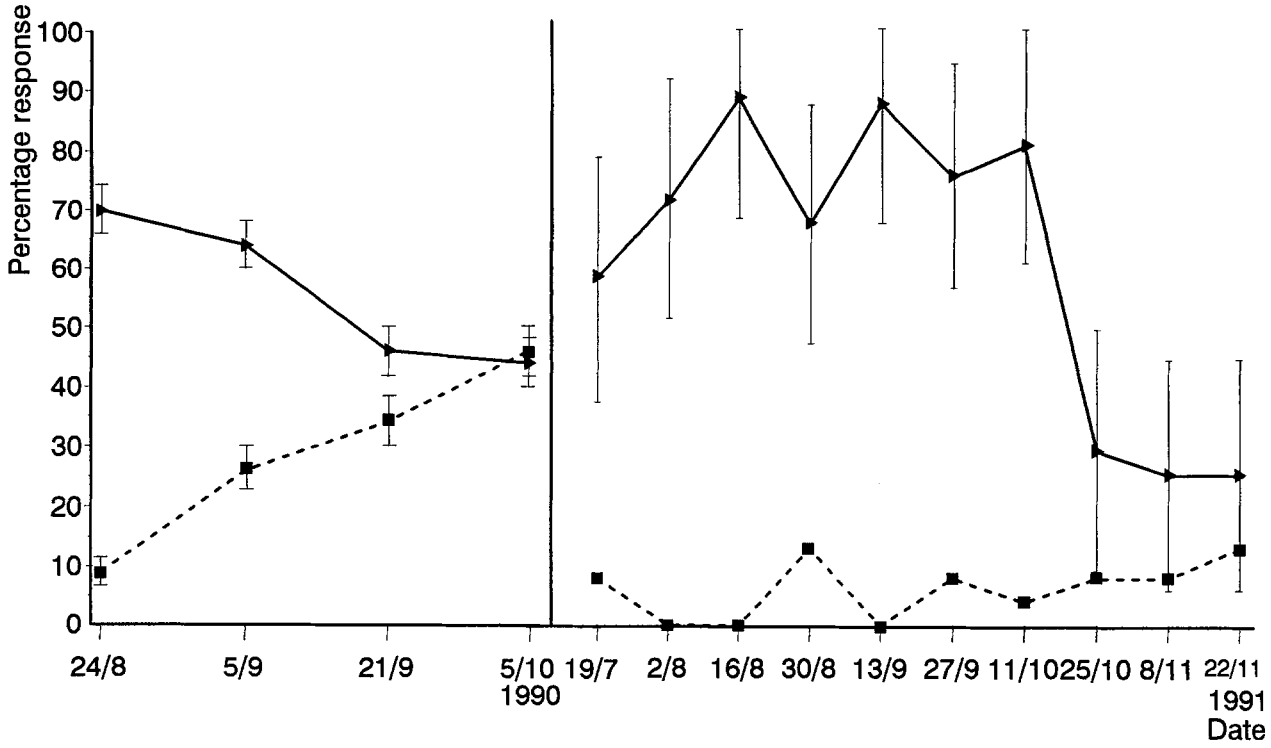

Table 4. Effect of time spent on medium with TDZ upon the mean proportions (± s.e.) of cultures that established as shoot cultures following transfer to micropropagation medium expressed both as a percentage of the initial hypocotyl explants and as a percentage of the hypocotyl explants that produced shoots with TDZ. Means were obtained from three replicate experiments.

Period on medium with TDZ (days)	Percentage of cultures that died on medium with TDZ	Percentage of initial explants that established as shoot cultures on micropropagation medium	Percentage of cultures transferred to micropropagation medium that established as shoot cultures
0	1±1.3	19±4.7	20±4.7
7	0	55±5.9	55±5.9
14	11±3.7	65±5.6	73±5.5
21	6±2.7	65±5.6	69±5.6
28	21±5.0	58±6.1	74±6.1
35	19±4.7	74±5.3	91±3.8
LSD 5%	11.8	17.4	16.7
(10 d.f.) 1%	16.8	24.7	23.4
0.1%	24.3	35.8	34.4

Micropropagation of adventitious shoots

Adventitious shoots obtained from embryo hypocotyls with TDZ only elongated after transfer to micropropagation medium [DKW with 5.0 mg l^{-1} BAP; Hammatt and Ridout (1992)], where they proliferated into shoot cultures following the outgrowth of axillary shoots. In order to maximize the proportion of hypocotyl explants that ultimately gave rise to shoot cultures, hypocotyls from dried embryos were cultured on MS medium with 0.1 mg l^{-1} TDZ for periods of 0, 7, 14, 21, 28 and 35 d, followed by 28d on micropropagation medium (Table 4). When the number of shoot cultures obtained from three replicate experiments was expressed as a proportion of the initial explants, the effect of time on regeneration medium upon shoot culture production was strongly significant ($p < 0.001$), but this was mostly due to the difference between time 0 (hypocotyls placed directly onto micropropagation medium) and the other times. If this is removed from the analysis, there was no significant difference between the remaining treatments. When the number of shoot cultures produced is expressed as a proportion of the number of cultures transferred to micropropagation medium, there was also a significant effect of time spent on regeneration medium ($p < 0.001$), even if explants placed directly onto micropropagation medium (time 0) are excluded. The proportion of cultures that died increased with time spent on medium with TDZ ($p < 0.001$).

Shoots from micropropagation rooted in half-strength, hormone-free Woody Plant medium containing 1.0 mg l^{-1} IBA (Hammatt and Ridout, 1992) at rates varying from 0-80% depending on the genotype of the original embryo.

Discussion

This paper describes methods that resulted in adventitious regeneration of shoots and whole plants from hypocotyls of *F. excelsior*. Regeneration was improved using TDZ rather than BAP. Thidiazuron has been useful in obtaining adventitious shoot formation in a number of other woody plants including apple (Wallin and Johansson 1989), *Rhododendron* (Preece and Imel 1991), quince (Dolcet-Sanjuan *et al.* 1991) and elm (Bolyard *et al.* 1991). Comparisons between TDZ and BAP with quince (Dolcet-Sanjuan *et al.* 1991), and between TDZ and isopentenyladenine with *Rhododendron* (Preece and Imel 1991) have also shown that TDZ is more effective than adenine-based cytokinins in inducing adventitious shoots. Results with ash confirm previous findings with other woody species, that TDZ concentration also affects regeneration.

Supplies of fresh ash seed are only available for three months per year and seed set from year to year is unpredictable. It is encouraging therefore that shoots could also be obtained from embryo hypocotyls from storable, dried seeds, since this source can provide an alternative supply of explants when fresh seeds are unavailable. Results with both fresh and dried seeds suggested that MS medium with 0.1 mg l^{-1} TDZ resulted in good levels of regeneration and low levels of vitrification of the resulting shoots. The results also suggest that there could be some benefit from incorporating IBA with TDZ.

It is hoped that these results, with tissues from a range of genotypes, will form the basis of techniques for the regeneration of shoots from clonal explants such as stem sections and leaves of adult trees of proven field performance.

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