Vegetative Propagation Techniques for Oak, Ash, Sycamore and Spruce

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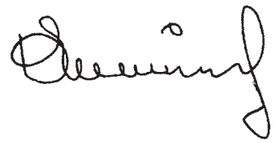
FOREWORD

Irish forest nurseries produce 80 million plants each year. Conifers account for the bulk of production - 67 million plants - but the proportion of broadleaves is ever increasing and they account for the balance of 13 million plants. *Growing for the Future* – the government's strategy for the development of the forestry sector has set a target afforestation rate of 20,000 hectares each year to 2030, 30% of which will be comprised of broadleaves. It is vital that the programme is underpinned by the use of quality, vigorous planting stock.

Planting stock quality contributes significantly to the return on the forestry investment. The investigation of techniques to improve planting stock quality has been an integral part of the COFORD R&D programme since 1995. Vegetative propagation using rooted cuttings is growing in popularity as a means to rapidly introduce genetically improved planting stock. Other related techniques are being developed which may have application in both coniferous and broadleaved species. These include somatic embryogenesis and photoautotrophic micropropagation. Both are at the early stages of development but they offer considerable advantages in rapidly deploying superior genetic material.

COFORD has funded research on vegetative propagation in a number of projects since 1995. Under the programme, methodologies for producing quality planting stock material have been refined and further developed. While further work is necessary to improve the cost-efficiency of such methods, superior planting stock is now being made ready for field testing under the current COFORD programme. This phase will demonstrate how successful the development work has been.

In conclusion I would like to acknowledge the dedication and commitment of the teams of researchers involved in the projects reported here. The results of the research will be of considerable interest to the forest nursery sector now and into the future.



David Nevins
Chairman

November 2001

SOMATIC EMBRYOGENESIS IN SITKA SPRUCE AND OAK

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SUMMARY

Somatic embryogenesis presents a potential method for the rapid propagation of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and oak (*Quercus* species). In Sitka spruce, initiation of embryogenic cell lines is the main bottleneck (about 4% of the embryos cultured formed embryogenic cell lines) and all modifications of the initiation process have failed to significantly increase initiation rates. However, once embryogenic cell lines are established, somatic embryos can be formed, matured, germinated and converted into complete plants. A preliminary analysis of the estimated production costs has identified the handling of individual plants as the most costly step in the process.

In oak the situation is slightly different because while initiation rates can be up to 27%, it is the switching off of the production of somatic embryos that presents the greatest bottleneck.

Nevertheless, both systems, even in their present state of development, are capable of producing small numbers of plantlets for establishment in field trials. Further work is necessary to improve the efficiency of the process and to prove the genetic and physiological uniformity and quality of the plant material produced. Larger field trials are needed to demonstrate the performance of material propagated by this method.

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1. INTRODUCTION

The irregularity associated with the periodicity of flowering and seed production in the breeding and production of genetically improved material results in significant delays in putting the results of tree breeding efforts into practical use. Vegetative propagation techniques such as rooting of cuttings, grafting, air layering and micropropagation offer a way to avoid these delays. In vegetative propagation the cost of producing plants is critical. Because forest tree seedling costs range from £0.08 (€0.10) to £0.25 (€0.30) each, it is very difficult to produce vegetatively propagated material at competitive prices. As a result, grafting and air layering are too expensive to employ in large-scale propagation. Rooted cuttings of forest trees typically cost two or more times the cost of seed propagated material because of the labour required. Even if every cutting roots, which is usually not the case, this produces only one plant. The advantage of micropropagation is that each culture can produce an unlimited number of plants, at least in theory.

The problem with conventional micropropagation systems that depend on organogenesis is that the amount of labour (handling) involved in producing the plants results in high production costs. In organogenesis the process involves culture initiation, stimulation of axillary or adventitious buds, elongation of buds into shoots, excision of shoots, rooting of shoots and transfer from culture to the greenhouse, each step requiring handling. The cost of Monterey pine (*Pinus radiata* D. Don.) plants propagated by organogenesis was estimated at between five to six times the cost of conventional seedlings (Smith 1991).

In somatic embryogenesis the shoot and root pole of the plant are formed in one step rather than two separate steps. Because it involves fewer steps and therefore requires less labour, somatic embryogenesis could produce plants at costs that may be similar to rooted cutting costs or even approach seedling production costs. For these reasons somatic embryogenesis was tested as a propagation method for Sitka spruce and oak.

The basic steps in the process of somatic embryogenesis are:
- initiation of embryogenic cell lines;
- growth and maintenance of cell line;
- somatic embryo formation;
- maturation of somatic embryo;
- germination of somatic embryo;
- conversion of somatic embryo into a plant.

For a review of the methods for production of somatic embryogenesis the reader is referred to a review of the conifers by Tautorus *et al*. (1991) and of oak by Wilhelm *et al*. (1999).

2. SITKA SPRUCE SOMATIC EMBRYOGENESIS

Sitka spruce embryogenesis involves the following four steps - initiation, maintenance/proliferation, maturation and germination.

2.1 Materials and methods/results

2.1.1 Initiation

Mature seeds from progeny tested families were used to initiate embryogenic cultures. Seeds were surface sterilised in a 20% bleach solution containing several drops of Tween 20 as a wetting agent for 20 minutes followed by three rinses in sterile, distilled water. Approximately 100 zygotic embryos were dissected from the seeds and were cultured on a basal induction medium consisting of a modified Murashige and Skoog (MS) mineral medium supplemented with 2,4-D (1.0 mg L^{-1}), BAP (0.5 mg L^{-1}) and kinetin (0.5 mg L^{-1}) (Appendices I and II). The medium contained 30 g L^{-1} of sucrose and was solidified with 6 g L^{-1} agar. All cultures were maintained in the dark at 25 ˚C for 2 months before examination.

Embryogenic tissue in conifers is not a typical unorganised or 'callus' type of tissue (Tautorus *et al*. 1991). It consists of two distinct, organised cell types; the small meristematic cells of the embryogenic 'heads' and the large, elongated suspensor cells. These two cell types are very similar to the types of cells that form zygotic embryos in developing ovules. Thus, to refer to the tissue that produces somatic embryos as 'callus' or even 'embryogenic callus' is incorrect because the tissue is highly organised and consists of very highly specialised cells. The term 'embryogenic suspensor mass' or ESM has also been suggested to distinguish this unique type of tissue.

In the first year of the project over 4,000 mature zygotic embryos (representing 27 selected families and a control) were used to initiate embryogenic cultures. Twelve of the families failed to show any response, eight families developed non-embryogenic callus and seven families initiated embryogenic cell lines in culture. These low rates were probably, in part, due to poor quality, ageing seed. In later work, a total of 15 embryogenic cell lines were established with induction frequencies (expressed as a percentage of the initial number of zygotic embryos cultured) ranging from 0.9 to 4.8%. Among the families that formed embryogenic cell lines were families 191, 574 and 587, which are some of the best performing families in Coillte's Sitka spruce improvement programme.

In another initiation study, seedling cotyledons (the first seedling leaves) were tested as an explant source. One cotyledon was removed from each seedling (100/family). Four families were tested (251, 574, 589 and 183). Embryogenic tissue was obtained from only one family (589) and the initiation frequency was very low (1%). Needles were also removed from some plants produced by somatic embryogenesis of family 183, but no embryogenic tissue was produced.

In the second year of the project, six of the same families were again initiated in culture. In contrast to the first year's results, only one family (574) developed embryogenic cultures (Table 1). The viability of the seed being used was tested by sowing it in compost. Only one family (574) was still viable.

TABLE 1: INITIATION OF EMBRYOGENIC CULTURES FROM MATURE ZYGOTIC EMBRYOS OF SIX SELECTED FAMILIES.

Family	*Number of embryos cultured*	*Sterile seeds in culture* %	*Embryos with callus development* %	*Number of embryogenic cultures*	*Initiation frequency* %
191	420	80	0	0	0
574	400	83	68	2	0.6
580	100	30	0	0	0
587	427	92	60	0	0
589	490	36	61	0	0
576	400	95	15	0	0

TABLE 2: INITIATION OF EMBRYOGENIC CULTURES FROM FIVE COMMERCIAL SEED LOTS.

Family	*Number of embryos cultured*	*Sterile seeds in culture* %	*Embryos with callus development* %	*Number of embryogenic cultures*	*Initiation frequency* %
V130	200	84	100	1	0.6
V131	150	86	100	3	2.3
W62	100	98	100	1	1.0
W17	100	96	100	3	3.1
SQ	100	50	100	2	4.0

Embryos from five commercial seed lots were initiated in culture to compare initiation frequency with that of selected families (Table 2). Initiation frequencies ranged from 0.6 to 4%, which were similar to results obtained with selected seeds in the first year of the project.

A range of experiments, with the specific aim of improving initiation frequency from mature embryos, was carried out. The standard initiation medium with various modifications was used. Twenty-five embryos from each seed source were used for each treatment.

The effect of carbohydrate source and concentration was tested (Table 3). Replacing sucrose with maltose resulted in the development of adventitious shoots. These shoots were transferred to MS medium where they continued to develop. Sucrose concentrations of 1 to 4% were tested. The best treatment was 1% sucrose (10 g L^{-1}).

The use of activated charcoal (1.25 g L^{-1}) in the initiation medium stimulated embryo germination rather than embryogenesis. All roots, hypocotyls and cotyledons were transferred back to fresh initiation medium to try to induce embryogenic cultures. None were obtained (data not presented).

Various concentrations of the auxins 2,4-D (0.5 to 16 mg L^{-1}), NAA (0.5 to 6.0 mg L^{-1}) and Picloram (0.25 to 2.0 mg L^{-1}) were tested for their ability to initiate embryogenic tissue (Table 4). Cultures were successfully initiated with 2,4-D at 2.0 mg L^{-1} in one of the two families tested. Higher initiation frequencies were obtained with NAA. The optimum concentrations of NAA were in the range of 1.0 to 4.0 mg L^{-1}.

The effect of gelling agent and concentration was tested (agar at 7, 10, 15 g L^{-1} and phytagel at 2, 4, 6, 8 g L^{-1}). No effect on initiation was observed (data not presented).

The effect of varying the concentrations of NH_4Cl and $NaNO_3$ is known to influence the formation of embryogenic tissue in other plant species. Trials with Sitka spruce resulted in embryogenic cultures developing at both high and low concentrations of nitrate (Table 5). Further work on the effect of nitrate, ammonium and total nitrogen concentration on somatic embryogenesis needs to be carried out.

A medium, (BLG medium, Brown and Lawrance glutamate), in which the inorganic ammonium nitrogen is replaced by an equivalent amount of organic nitrogen in the form of glutamine was tested. A high initiation frequency was reported to

TABLE 3: EFFECT OF CARBOHYDRATE TYPE AND CONCENTRATION ON EMBRYOGENIC INITIATION (25 EMBRYOS/TREATMENT).

Treatment/family	*Concentration/initiation frequency*			
Sucrose	10 g L^{-1}	20 g L^{-1}	30 g L^{-1}	40 g L^{-1}
574	8%	0%	0%	0%
251	8%	0%	0%	0%
Maltose	30 g L^{-1}	Stimulation of adventitious shoots		
191	0%			
574	0%			
587	0%			
576	0%			

TABLE 4: EFFECT OF GROWTH REGULATOR TYPE AND CONCENTRATION ON INITIATION (25 EMBRYOS/TREATMENT).

Treatment/family	*Concentration/initiation frequency*							
2,4-D	0.5 mg L^{-1}	1.0 mg L^{-1}	2.0 mg L^{-1}	4.0 mg L^{-1}	6.0 mg L^{-1}	8.0 mg L^{-1}	12.0 mg L^{-1}	16.0 mg L^{-1}
251	0%	0%	0%	0%	0%	0%	0%	0%
W62	0%	0%	4%	0%	0%	0%	0%	0%
NAA	0.5 mg L^{-1}	1.0 mg L^{-1}	2.0 mg L^{-1}	4.0 mg L^{-1}	6.0 mg L^{-1}			
251	0%	4%	4%	0%	0%			
574	0%	4%	0%	8%	0%			
V87	0%	0%	8%	0%	0%			
W62	0%	0%	0%	0%	0%			
Picloram	0.25 mg L^{-1}	0.5 mg L^{-1}	1.0 mg L^{-1}	2.0 mg L^{-1}				
574	0%	0%	0%	0%				
251	0%	0%	0%	0%				

occur in Norway spruce (*Picea abies* (L.) Karst.) when embryos were cultured on BLG medium in the light (Verhagen and Wann 1989). No embryogenic cultures were obtained however, when this medium was tested with Sitka spruce (data not presented).

The embryogenic initiation rates achieved in Sitka spruce are very low (maximum frequency observed in this work was 12%) and none of the treatments tested appear to result in a significant improvement in the initiation rate. All of the work presented above has been done with mature zygotic embryos serving as the starting material. Perhaps through the use of immature zygotic embryos as the original explants, the frequency of embryogenic initiation can be increased, as has been demonstrated in Norway spruce.

2.1.2 Maintenance

Embryogenic cell lines were maintained on the same nutrient medium as that used in the initiation of embryogenic cell lines. Cultures were maintained on agar solidified media in petri dishes in darkness at 25 ˚C and were subcultured every two weeks.

Cell lines established in the first year of the project were maintained in the above mentioned manner and used for later experiments. Concerns about possible loss of embryogenic potential over time led to investigations of alternative ways to maintain embryogenic cell lines. Cold storage was one such approach. Fifteen cell lines were placed in a cold store (+4 ˚C). After 8 months all cultures were removed and transferred to fresh maintenance medium and placed in the growth room. Three subcultures later, only two cell lines (251I and 251B) showed signs of growth or embryogenic tissue. Later, only line 251I successfully regenerated emblings. Thus, cold storage of embryogenic cell lines does not appear to be a viable option, at least at the temperatures and conditions tested.

2.1.3 Plantlet production

Somatic embryos of Sitka spruce developed by transferring embryogenic tissue from the

TABLE 5: THE EFFECT OF VARYING CONCENTRATIONS OF NH_4Cl AND $NaNO_3$ ON THE FREQUENCY OF EMBRYOGENIC CULTURE INITIATION (25 EMBRYOS/TREATMENT).

		NH_4Cl			
	Family 574	1.5 mM	2.5 mM	5.0 mM	7.5 mM
$NaNO_3$	1.5 mM	12%	0%	0%	0%
	2.5 mM	0%	0%	0%	0%
	5.0 mM	0%	0%	12%	0%
		NH_4Cl			
	Family 251	1.5 mM	2.5 mM	5.0 mM	7.5 mM
$NaNO_3$	1.5 mM	0%	0%	0%	0%
	2.5 mM	0%	0%	0%	0%
	5.0 mM	0%	0%	4%	0%

TABLE 6: EFFECT OF FAMILY AND CELL LINE ON THE ABILITY OF SITKA SPRUCE EMBRYOGENIC CELL LINES TO FORM SOMATIC EMBRYOS.

Cell line	*Average number of somatic embryos per gram of embryogenic tissue*
251H	4.0
251I	7.9
251J	2.5
12C	7.5
574A	27.0
576B	6.7

maintenance medium to a modified MS medium containing abscisic acid (ABA) at 50 mg L^{-1} and activated charcoal (1.25 g L^{-1}). Cultures are maintained on this medium for 2 months in the dark during which somatic embryos develop and mature. They are then transferred to a modified MS medium containing no exogenous growth regulators and placed in the light for somatic embryo germination and the production of emblings. The effect of different genetic backgrounds on the ability to produce somatic embryos can be seen from a comparison of 6 different cell lines originating from four different Sitka spruce families (Table 6).

Not only were differences between families seen, but also differences between cell lines originating from different zygotic embryos of the same open pollinated family (251) were observed.

Methods to improve plantlet production focused on the composition of the maturation medium. Maturation of somatic embryos results in well developed shoot and root apical meristems that will elongate simultaneously when 'germination' occurs. Initial experiments were run testing the effect of different osmotic treatments to improve somatic embryo production. Various osmotica were tested, including sorbitol (3%), mannitol (2 and 4%) and polyethylene glycol (3, 6 and 9%). Five different embryogenic cell lines were tested, but none of the above treatments had any significant effect on embryo regeneration (data not presented).

The effect of phytagel (a compound used to solidify the nutrient medium similar to agar) concentration yielded some interesting results (Figure 1). Concentrations of 2 to 10 g L^{-1} were tested and compared to the standard treatment containing 6 g L^{-1} agar. Six lines were tested. The results presented were the means of two experiments. Although the results do not conclusively show that one individual treatment was best, in almost all cases the standard treatment produced the fewest number of cotyledonary

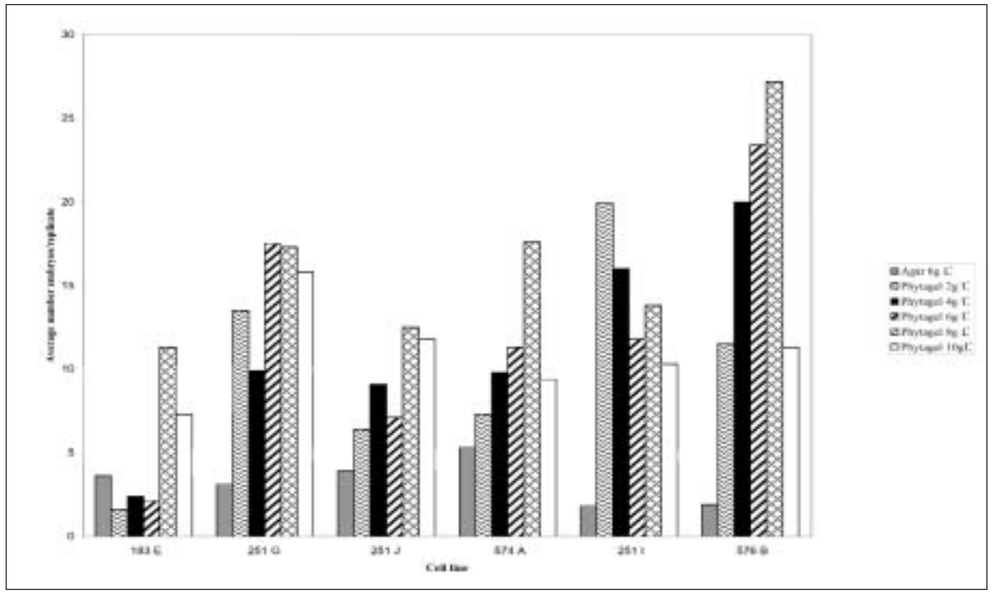

FIGURE 1: THE EFFECT OF PHYTAGEL CONCENTRATION ON MATURATION OF EMBRYOS.

embryos. Results indicate that a higher number of embryos may be obtained by using phytagel instead of agar in the maturation medium. In addition, it would appear that an increase in embryo number occurs with an increase in phytagel concentration, up to 8 g L^{-1}.

The effect of phytagel concentration in the maturation medium on subsequent germination was also tested. No significant difference in germination rate was observed between embryos matured under different phytagel concentrations (data not presented). Germination rates for cell lines ranged from 0 to 20%. These results are lower than previous years and may indicate a decline in the performance of the cell lines (maintained for 3 years in culture before being used in these experiments). No distinct differences in embryo morphology were observed between treatments, except that embryos tended to be smaller at higher phytagel concentrations.

In a subsequent experiment, the effect of varying the agar concentration on maturation was tested (Figure 2). Results obtained indicated a decrease in embryo number development with increasing agar concentration. The effect of these treatments on subsequent germination could not be determined because germination rates were zero.

These results show that there are fundamental differences between agar and phytagel in the production of somatic embryos. In most work the gelling agent is assumed to have no role in the tissue culture process, while these results illustrate that this is clearly not true in production of embryogenic Sitka spruce cultures.

The gaseous plant growth regulator ethylene has been shown to play a role in the development of somatic embryos. Therefore the effect of silver nitrate (an inactivator of ethylene effects) in the maturation medium was tested (Figure 3). Results show that in some instances the standard maturation medium performed best, while in others the addition of silver nitrate was beneficial. Overall, it is felt that the inclusion of silver nitrate in the maturation medium failed to improve maturation.

Work aimed at improving the germination potential concentrated next on subjecting embryos to various desiccating treatments (Figure 4). The various treatments tested were agar 1% and sucrose 1.5% in the germination medium. In addition, embryos were desiccated under high humidity using the well system. This uses a 25 cell plastic multi-well plate with all of the outer 16 and the central well filled half way with water. Somatic embryos were placed in each of the remaining eight wells and exposed to this treatment for 3 weeks before being transferred to solidified germination medium. All treatments were compared to the standard treatment. Results are presented as the mean of two experiments (Figure 4). The least effective treatment was sucrose 1.5% for all cell lines tested. There were no significant differences between any other

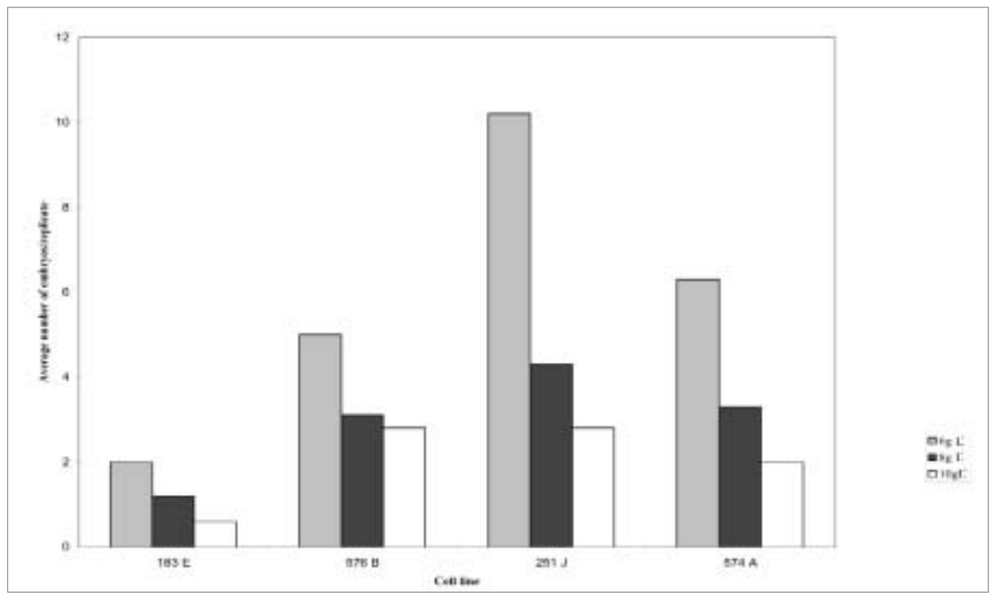

FIGURE 2: THE EFFECT OF AGAR CONCENTRATION ON MATURATION OF EMBRYOS.

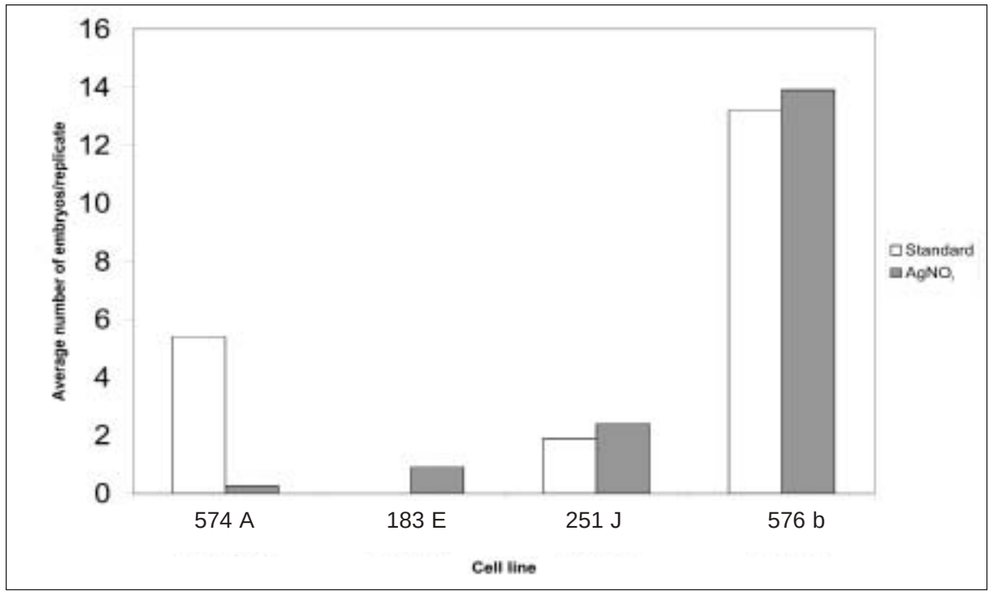

FIGURE 3: THE EFFECT OF SILVER NITRATE ON MATURATION OF EMBRYOS.

treatments except in cell line 576B where the standard did not perform as well.

Matured somatic embryos 'germinate' (simultaneous elongation of the shoot and root meristems) and produce a normal looking plantlet. Germinated somatic embryos need to be transferred from the low light levels and high humidity of the petri dish in the growth room to the higher light levels and lower humidity of the greenhouse. During this transition period the emblings become autotrophic. This process is known as 'acclimatization'. This is accomplished by potting the well-developed embling (with at least 5 mm of new root and growth of the true shoot above the cotyledons) in a 50:50 mixture of peat:perlite under a plastic propagating cover. After 4 to 6 weeks the humidity is gradually

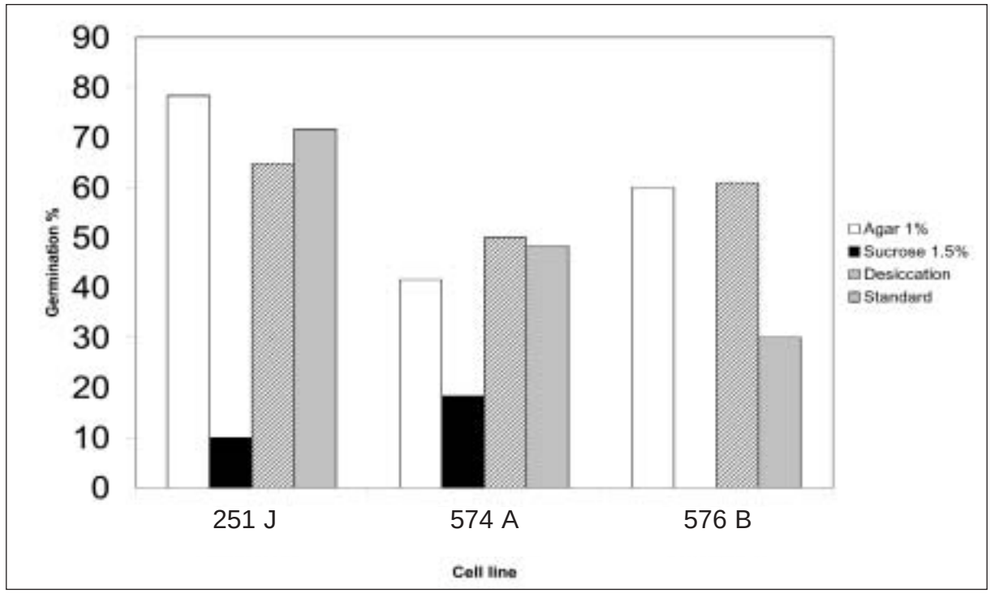

FIGURE 4: THE EFFECT OF DESICCATION TREATMENT ON EMBRYO GERMINATION.

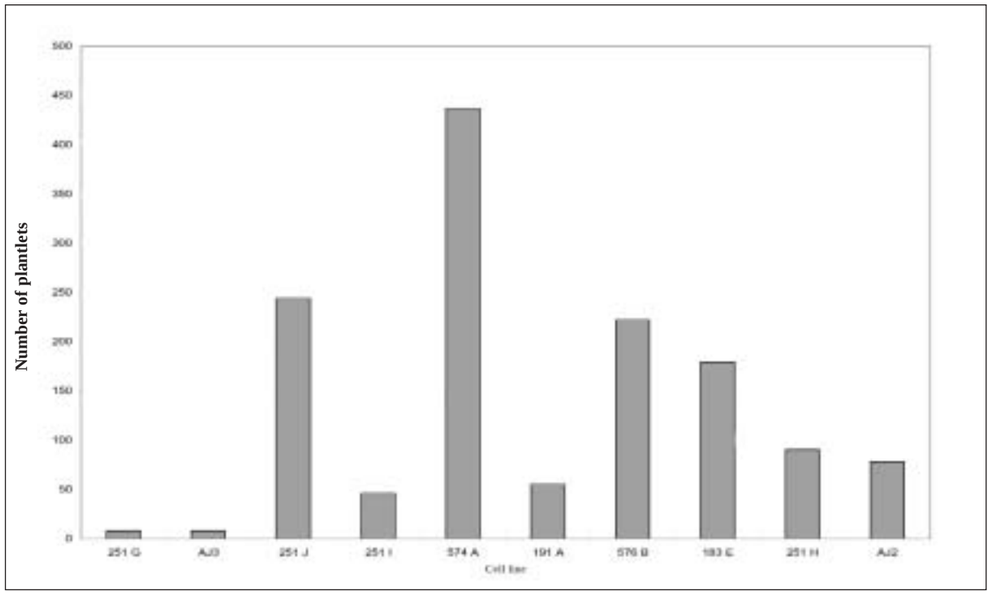

FIGURE 5: THE NUMBER OF PLANTLETS SUCCESSFULLY TRANSFERRED TO COMPOST.

reduced until the propagating cover can be completely removed at which time the emblings can be grown under normal greenhouse conditions. During 1997 about 1,367 plants were successfully established in compost (Figure 5). The percentage of emblings surviving ranged from 90 to 100%.

2.1.4 Embryogenic Suspension Cultures

Embryogenic cells can also be grown in a liquid medium (the same composition as the induction/maintenance medium but without agar or phytagel) on a rotary shaker to aerate the cells. Such suspension cultures can provide information on the growth of the different types of cells (embryogenic and suspensor cells), which is more difficult to assess in cultures maintained on solidified media. Shake flask experiments were set up with Sitka spruce embryogenic cell lines to analyse cell growth characteristics, optimum subculturing frequency and nutrient utilisation. Cells were grown for 14 days and analysed on days 0, 3, 7, 10 and 14. Settled cell volume (SCV), fresh weight (FW), dry weight (DW), pH and embryo numbers were recorded on each sampling day. Various technical problems were encountered during several attempts to run this experiment. Therefore, while no useful results were produced during this project, the technique is worth further evaluation. Embryogenic cell suspensions could be grown in a 'bioreactor' where fresh nutrients are added periodically to produce large volumes of embryogenic cells that could be induced to form somatic embryos for large-scale plantlet production.

2.1.5 Production costs

To identify the most costly steps in the somatic embryogenesis process, the current non-optimised process was subjected to an economic analysis. Because of the low initiation frequency all costs were based on the use of an established embryogenic cell line. The production is divided into two phases, the *in vitro* and the plantlet or embling phase. The *in vitro* costs began with a single culture that had to be grown to produce enough tissue to produce 1,000 plates of embryogenic tissue. All the *in vitro* costs are based per 1,000 petri plates. Once this had been achieved and emblings had been regenerated the costs were based on the cost to produce 1,000 emblings.

All of the above estimates are based on the current non-optimised production method. The main point of note is that the most expensive single step is not in the *in vitro* somatic embryogenesis production steps (embryogenic tissue production, maturation or germination steps) as might be expected, but rather in the selection and transfer of

TABLE 7: ESTIMATED PRODUCTION COST OF THE STEPS IN THE PROCESS OF SITKA SPRUCE SOMATIC EMBRYOGENESIS.

Stage of production	*Cost*
Embryogenic tissue production	€546/1,000 plates
Maturation step	€411/1,000 plates
Germination step	€411/1,000 plates
Select and transfer emblings	€673/1,000 plates
Total in vitro embling production costs	€2,042/1,000 plates
Total in vitro costs	**€165/1000 emblings**
Cost to establish emblings in the greenhouse	€36/1,000 emblings
Yield sub-total (assume 90% survival)	**€223/1,000 emblings**
Overheads (20%)	€44/1,000 embling
Total cost at the end of the greenhouse phase	**€268/1,000 emblings**
Cost to grow 1 year in container	€63/1,000 emblings
Total cost of emblings ready to go to the field	**€331/1,000 emblings**

single germinating embryos. This is because this is the only stage in the process where single emblings are handled. Prior to this, only masses of embryogenic cells and clusters of developing emblings (consisting of tens to hundreds of somatic embryos) are handled *en mass*. When it comes to selecting out and handling individual embryos the production costs increase dramatically. Labour costs account for the largest percentage of the total production costs. Thus, the assumption that producing large numbers of embryogenic cells or indeed small plantlets in bioreactors (somatic embryogenesis production steps) will reduce plant production costs may not be correct. When plants are removed from the bioreactor they will still have to be handled separately, which will be the most costly step in the process. If a reduction of production costs of *in vitro* produced plant material is required, then methods for the automated or semi-automated selection and handling of plantlets should be developed.

A second important point is the overall cost of 1,000 emblings. A cost of €331/1,000 emblings is high compared to traditional unimproved Sitka spruce costing about €127/1,000, but it is more comparable to the cost of genetically improved rooted cuttings, which cost €235/1,000. It is also important to remember that these are based on a non-optimised production process. This cost is not as high as some estimates of the cost of micropropagated trees (propagated by organogenesis) that have been suggested {five to six times the cost of conventional seedlings (Smith 1991)}. With further research, the process can certainly be improved. Production costs will only decrease to equal the cost of rooted cuttings and may even approach the cost of producing seedlings.

2.1.6 Plantlet quality

Two embryogenic cell lines of the same family were used to regenerate emblings for field trials. These were acclimatized in the greenhouse. Seedling controls of the same family were grown in the same containers, in the same media and under the same greenhouse conditions as the emblings. Table 8 provides morphological comparisons between the emblings and the seedlings.

There were no significant morphological differences between emblings and seedlings of the same family except that the number of seedlings that had undergone a second flush during the autumn of 1998 was greater than either of the two embling lines. Because late flushing is considered a characteristic of young plants that decreases with increasing age, this observation would suggest that the emblings might have a more mature growth habit than seedlings. In addition, the Sitka emblings had a bluer needle colour than the seedlings, which may also be indicative of a more mature plant. Whether these differences were due to differences in the maturation state of the emblings and seedlings and whether these differences persist will need to be documented in the field trials.

In similar morphological comparisons between rooted Sitka spruce cuttings with seedling transplants there was a statistically significant difference in the number of branches, with the cuttings having fewer branches than the seedlings (Fennessy *et al*. 2000). The results presented here of comparisons between emblings and seedlings do not show such a trend.

TABLE 8: MORPHOLOGICAL COMPARISONS OF EMBLINGS OF FAMILY 574 (20 PLANTS/PLANT TYPE) MEASURED AT THE END OF THE 1998 GROWING SEASON.

Parameter	*574A emblings*	*574B emblings*	*Seedling controls*
Height cm	30.10	47.40	35.20
Stem diameter mm	3.05	3.75	3.05
No. of branches	11.60	16.00	15.60
Branches/height	0.38	0.34	0.44
Flushed %	0.00	0.00	42.00

2.1.7 Field trials

About 2,000 emblings from a range of families were acclimatized, grown in the greenhouse and examined in field trials.

2.2 Conclusions

In Sitka spruce somatic embryogenesis, initiation of embryogenic cell lines is the main bottleneck. All attempts to increase the initiation frequency have not been successful. There are two ways around this problem. First, by culturing large numbers of embryos a small number of embryogenic cell lines will always be initiated. A second approach, not tested in this work, would be to use immature zygotic embryos where, in species such as Norway spruce, initiation rates are higher than with mature zygotic embryos. This is probably the best solution to the problem of low initiation rates.

Development of a method to maintain embryogenic cell lines in a state where they will not deteriorate is important. Cold storage (+4 ˚C) did not appear to be successful. Storage of cells cryogenically at liquid nitrogen temperature (-196 ˚C) has been successful with other cell lines, but it requires equipment to freeze the cells at a constant rate (1 ˚C/minute) and a liquid nitrogen storage container, both of which were not available for this study.

Somatic embryo formation does not present a problem, provided the lines have been well maintained in culture. The use of ABA and the high humidity desiccation treatment are reasonably successful methods for the production of mature somatic embryos capable of good germination rates. Germination rates (about 25%) could be further improved, but conversion rates of 90% are very acceptable. Even the current non-optimised state-of-the-art process is capable of producing enough emblings for the establishment of field trials to demonstrate the potential of the method.

Although the process would have its ultimate application in the production of plant material for use in the field, this is probably a number of years away. The most likely immediate application of this technology will be the propagation of selected families for use as stock plants to produce cuttings for rooting. Later, with further improvements in technology and reductions in the production costs it may be possible to produce emblings that will go directly to the field. Encapsulation of somatic embryos in a coating could result in ‘artificial seeds’ that could be handled exactly like zygotic seeds.

3. OAK SOMATIC EMBRYOGENESIS

Oak somatic embryogenesis involves the same four main steps - initiation, maintenance/proliferation, maturation and germination as in the case of Sitka spruce.

3.1 Materials and methods/results

3.1.1 Initiation

Immature acorns (late July) from six oak trees were collected, surface sterilised and the embryos cultured on an induction medium consisting of a MS medium containing BAP 1.0 mg L^{-1}, sucrose 30 g L^{-1} and solidified with 6 g L^{-1} agar. Cultures were maintained in the dark at 25 ˚C. Between 30 and 50 immature zygotic embryos per tree were established in culture. Initiation frequencies ranged from 0.7 to 26.2%. In total 24 embryogenic cell lines were established in culture. The material used to initiate the cultures was from phenotypically selected individuals, but none of these trees has been progeny tested so they are of unproven genetic value.

In oak, somatic embryogenesis is achieved by the original zygotic embryo ‘budding’ to form a cluster of somatic embryos, which continue to 'bud' to produce more somatic embryos. The formation of an unorganised callus tissue or even an organised embryogenic tissue, as in Sitka spruce, does not occur. This technique can be described as secondary somatic embryogenesis in which the immature zygotic embryo is induced to replicate itself indefinitely.

The induction of oak somatic embryos from mature tree tissues or at least non-seed tissues such as leaf of stem segments has been reported. This technique would allow for the propagation of proven superior mature oak trees. During the course of this project, leaf and stem explants from micropropagated oak cultures initiated from mature trees, were cultured on a somatic embryogenesis induction medium. Sterile explants from shoot tip micropropagated oak lines were placed on MS medium {containing BAP (2.25 mg L^{-1}) and NAA (1.8 mg L^{-1})} and placed in the dark for 2 months. Non-embryogenic callus was formed from both the leaf and stem segments but no embryogenic tissue was ever observed in these cultures.

3.1.2 Maintenance

Once an embryogenic culture had been initiated it was maintained in culture on MS medium containing BAP (0.2 mg L^{-1}) and 3% sucrose. All cell lines were maintained in the light at 25 ˚C and transferred every 4 to 6 weeks.

3.1.3 Maturation/proliferation and germination

Maintenance of oak embryogenic cell lines was straightforward and presented no technical problems. The difficulty, however, was in the switching off of the process of secondary somatic embryogenesis and stimulating the somatic embryos to mature and then germinate. Various maturation treatments were tested including different nutrient media, hormone combinations and different desiccation methods. The most effective method was the use of a higher agar concentration (1%). Increasing the agar concentration effectively induces osmotic stress, which reportedly stops budding of somatic embryos and enhances maturation. After 5 weeks on the high agar medium the embryos were transferred either to a germination medium (basal medium without exogenous plant growth regulators) or subjected to a partial desiccation treatment (the same multi-well system described for desiccation of Sitka spruce somatic embryos). Partial desiccation also provides a trigger to stop budding of somatic embryos and to start the maturation programme.

Somatic plants have been obtained; however, no one treatment has consistently produced a regular supply of emblings. Seven emblings from cell line 431/H9 and ten from line 2D have been established in compost in the greenhouse. In addition emblings from cell line 2G (five emblings), 2D (three emblings) and 3C (three emblings) are developing in culture. The emblings growing in compost will be lined out in the nursery.

3.2 Conclusions

Having material to put into culture is the main limiting factor in oak somatic embryogenesis. Good acorn crops occur once every three to seven years and during the course of this project a good acorn crop occurred only in 1996. In addition, there are no tested, proven superior families of oak that would warrant the extra cost of vegetative propagation.

Maintenance of oak embryogenic cell lines is not a problem. One cell line that has been in culture since 1995 and has been maintained by subculture every six to eight weeks continues to produce good quality embryos after almost six years in culture. Similarly, formation of somatic embryos is also generally not a problem.

The main problem in oak somatic embryogenesis is turning off the embryo 'budding' programme and turning on the embryo development and maturation programme. This has also been a problem in other plant species where secondary somatic embryogenesis occurs. The high humidity desiccation treatment and the high agar treatment show promise in helping to overcome this bottleneck. Once mature somatic embryos can be produced, they can be germinated and transferred to the greenhouse where they appear to grow normally.

Finally, there is the question about the public acceptance of ‘clonal oak’. Nevertheless, this technique could be a way to accelerate the propagation of superior oak phenotypes once they have been identified in a tree improvement programme.

4. OVERALL CONCLUSIONS REGARDING SOMATIC EMBRYOGENESIS

Based on the results of this project, somatic embryogenesis continues to have great potential as a vegetative propagation method for both Sitka spruce and to a lesser extent oak. In both species, lack of the knowledge of the basic physiology and biochemistry of zygotic embryo development is the major bottleneck in the process. Nevertheless, a number of emblings of both Sitka spruce and oak have been successfully produced. Further work should concentrate on the genetic fidelity and uniformity over time of these emblings. The potential to use tissue from trees old enough to have demonstrated their superior characteristics to establish embryogenic cell lines could make true 'clonal forestry' a reality. The uniformity and reliability of clonal planting stock could have a significant effect on the production of quality material in plantation forests.

While further work is necessary to improve somatic embryo maturation techniques, the system, at least in spruce, is well enough understood to be able to produce sufficient material to establish small field trials of emblings produced by this method. The next step is to establish larger field trials of somatic embryos to demonstrate to foresters and the public the field performance of this material. Further research work, specifically on improving initiation and maturation rates as well as documenting the genetic fidelity and uniformity of the resulting emblings is necessary. This information should help overcome the concerns that foresters and the public may have concerning propagation by somatic embryogenesis.

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VEGETATIVE PROPAGATION OF SELECTED REPRODUCTIVE STOCKS OF ASH AND SYCAMORE

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SUMMARY

The feasibility of using vegetative propagation to provide plants from selected trees of ash (*Fraxinus excelsior* L.) and sycamore (*Acer pseudoplatanus* L.) was determined. Grafting was the first method used for propagating plants from the mature selected trees. All ash clones and 73% of sycamore clones were successfully grafted. Grafted plants were conserved in the nursery and field for further propagation.

Cuttings from grafted plants of sycamore gave 25% rooting. Cuttings taken from sycamore plants which had self rooted gave 49% rooting. This indicated that sycamore may have been rejuvenated and further improvements in rooting rates may be possible.

Micropropagation of sycamore was difficult and gave low propagation rates; cutting propagation rather than micropropagation is a more practical option for sycamore. Although ash gave poor rooting rates in cuttings, micropropagation of several selected clones was successful. The culturing of ash buds with high concentrations of cytokinins was necessary to establish viable cultures, but the regular transfer of cultures to cytokinin-free medium was necessary to maintain a healthy status in the cultures. In this protocol, spontaneous rooting in shoot cultures was observed and plants were successfully weaned to the glasshouse.

Established clones of ash were bulked up and are now available together with the method for large-scale vegetative propagation of selected lines of ash to provide plants for field testing.

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1. INTRODUCTION

Ash and sycamore are very important broadleaved species in Ireland. There has been little work undertaken so far on the genetic improvement of ash and sycamore. For many species, the use of seed from the most productive provenances can yield a 4-6% increase in genetic gain/volume over unselected material. The selection of the best performing individuals can give a further gain of 5-15%.

The basis for genetic improvement is to select material with a broad genetic base. Selected trees should also be near maturity since their growth and morphological performance will be known and also because the performance of juvenile trees is very difficult to predict with accuracy. In collaboration with Coillte, the most productive individual trees with a superior stem form at maturity were identified (selected 'elite' trees) in Coillte forests. Scions from the selected trees were grafted. The grafted plants were conserved to form a core that would be a foundation for further genetic improvement. The material could be used in two ways: to produce seed progeny or to produce plants by vegetative propagation. The vegetative propagation of selected trees allows the production of lines or varieties that are the exact genetic copy of the original tree and offer great potential to increase the productivity and value of new plantations once they have been field tested.

Cuttings collected from mature trees generally have a low rooting percentage. This is due to physiological changes that take place as the tree gets older. Micropropagation and grafting are potential methods to achieve vegetative propagation from mature selected trees. Alternative approaches are either to rejuvenate the selected plants or to use material from donor plants, which may exhibit some juvenile characteristics (one such characteristic is the capacity for rooting).

This project describes experiments aimed at establishing methods for the vegetative propagation of ash and sycamore using conventional cuttings and micropropagation of shoot cultures so that selected lines could be produced on a large scale. The rooting capacity was evaluated in cuttings taken from different sources of donor plants: mature trees, grafted plants, re-grafted plants, seedlings, hedges, self rooted cuttings and micropropagated plants.

2. MATERIALS AND METHODS

Approximately 100 elite ash and sycamore trees were selected. The trees were defined as "clean, with straight stems and butts, free from knots, as round as possible with the heart straight down the middle, free from wandering heart, with minimal taper and without star or ring shake or epicormic burrs".

Cuttings were collected from different types of donor plants as indicated. They were dipped in rooting powder (Seradix 3), except where indicated otherwise, and inserted into a substrate of $^{2}/_{3}$ peat and $^{1}/_{3}$ perlite in 8 x 5 celled Hassey trays. These were placed in an enclosed mist unit with a mist burst of 18 seconds duration every 30 minutes from 7 am to 8 pm daily. Rooting evaluations were made in October of each year.

For initiating shoot cultures, buds were sterilised by placement in 0.1% w/v mercuric chloride for 15 minutes, followed by one wash in sterile water, then a shake for 20 minutes in 7% w/v calcium hypochlorite and three rinses in sterile water. Single apical buds and nodes were cultured, one per petri dish (5.0 cm diameter, 20 mm deep). Buds were transferred to fresh medium every 4-5 weeks. When shoot elongation occurred, the shoots were cut in half and re-cultured. Later, explants were cultured consisting of: (i) a single apical bud, (ii) single nodes and (iii), multi-nodal explants consisting of at least two nodes, usually from the shoot bases which had 2-3 buds on short internodes. The basal medium (MS) for shoot culture was Murashige and Skoog (1962) with B5 Vitamins (Gamborg *et al*. 1968) instead of MS vitamins. The standard medium for ash shoot culturing (M9) contained thidiazuron (TDZ), 1.1 mg L^{-1}, indole butyric acid (IBA), 0.2 mg L^{-1}, benzyladenine (BA), 5.0 mg L^{-1} and sucrose 3% w/v. All media were agar solidified (Difco Bacto) 8.5 g L^{-1}, and pH adjusted to 5.8 before autoclaving. Shoots were initially cultured in petri plates and later in 150 ml glass powder jars with aluminium screw top lids with 35 ml of medium/jar and were transferred to fresh medium every 28 - 32 days. The light regime was 40 µEm^{2} sec^{-1} with a 16 hr-photoperiod and a temperature of 22 ˚C ± 2 ˚C.

3. RESULTS AND DISCUSSION

Following identification of elite trees, scions were collected and grafted onto 2-year-old rootstocks during the dormant period using a cleft graft. All 70 elite clones of ash gave viable grafts whereas with sycamore 73% of clones gave viable grafts (Table 1). Scions collected from the successful grafts gave an improvement in the graft viability with sycamore when grafts were made either in February or July (Table 1). The grafted elite trees are now field planted at Coillte's Kilmacurragh Nursery and at Kinsealy. In the Coillte nursery they are widely spaced for seed production. At Kinsealy they are planted closer together to form hedges from which cuttings can be taken and the material propagated and used in clonal field tests in farm/forestry.

There is little previous research on rooting cuttings from either juvenile or mature trees of ash. In this study, juvenile cuttings were tested to define some parameters affecting rooting. It is necessary to know the optimal conditions to achieve rooting in cuttings, so that the method can be applied on a larger scale to cuttings from selected elite lines. Cuttings from different sources of donor plants were used which may represent different states of physiological maturation corresponding to juvenile, mature and rejuvenated states.

3.1 Rooting in cuttings derived from seedlings and micropropagated plants

Table 2 summarises the rooting response of ash and the quality of roots formed with juvenile cuttings, which consisted of an apex plus two nodes. Auxin was mixed with lanolin as a paste and applied to the apical buds of the cuttings. Application of auxin appeared to stimulate rooting. This suggests that the applied auxin was capable of being absorbed at the apex and of stimulating rooting at the base of the cutting. Without auxin treatment, 65% of the cuttings rooted whereas 92% rooting was achieved with 8000 mg L^{-1} IBA. In this case, 90% of the rooted cuttings had a strong rootball. The appearance of roots is shown in Figure 1.

TABLE 1: VIABILITY OF GRAFTS OF ELITE ASH AND SYCAMORE WITH DIFFERENT SOURCES OF SCIONS AT DIFFERENT TIME PERIODS.

	Origin of scions	*Grafting date*	*[2]Mean of clone viability %*	*Mean of graft viability %*
Ash	mature tree	Feb	100 (70)	97
	grafted tree	July[1]	100 (20)	85
Sycamore	mature tree	Feb	73 (70)	25
	grafted tree	Feb	100 (20)	58
	grafted tree	July[1]	73 (16)	45

[1]Scions grafted by 'tube' method (Douglas *et al*. 1996).
[2]Numbers in parenthesis indicate the number of clones tested.

TABLE 2: EFFECT OF AUXIN TYPE AND CONCENTRATION ON ROOTING IN JUVENILE CUTTINGS OF ASH.

Auxin[1] treatment (mg L^{-1})	*No. of cuttings*	*Rooting class[2]*				*Total rooting %*
		0	*1*	*2*	*3*	
None	55	13	1	5	36	65
IBA (300)	55	14	0	11	35	84
IBA (1000)	60	18	0	5	36	68
IBA (2000)	55	18	1	4	31	65
IBA (4000)	58	29	0	6	23	50
IBA (8000)	49	14	0	1	44	92
NAA (2000)	50	17	4	4	33	82

[1]Cuttings with apex plus two nodes, auxin applied to the cutting apical bud.
IBA: indolebutyric acid, NAA: naphthalene acetic acid.
[2]Rooting class: 0: no roots, 1: roots 0.5 - 2.0 cm long, 2: roots 0.5 - 2.0 cm long and at least one root over 5 cm, 3: roots 0.5 - 2.0 cm long and two or more roots over 5 cm.

FIGURE 1: ROOTING IN SEEDLING CUTTINGS OF ASH.
Auxin (2000 ppm) applied to the cutting apex as in Table 2. Top: NAA treatment, bottom: IBA treatment.

The rooting capacity of two types of cutting was compared: apical 2-node cuttings with sub-apical and 2-node cuttings with different auxin treatments.

Without auxin, rooting was low at 34 - 44% (Table 3). The apical bud stimulated rooting but high rooting rates were also found in sub-apical two-node cuttings (75%). Seradix rooting powder (8000 ppm IBA in talc) was superior to the proprietary product 'Hormone Rooting Powder' (NAA 4000 ppm and 2% captan).

A subsequent experiment confirmed that high rooting rates could be achieved with sub-apical cuttings. Rooting in different substrates gave different rooting rates:

- $\frac{2}{3}$ peat + $\frac{1}{3}$ perlite gave 94% rooting in both apical and subapical cuttings;
- $\frac{2}{3}$ peat and $\frac{1}{3}$ peat nuggets (0-3 mm) gave 63% and 94% rooting in apical and subapical cuttings respectively;
- rooting was depressed with $\frac{2}{3}$ peat and $\frac{1}{3}$ peat nuggets (3-6 mm) and gave 55% and 75% rooting for apical and subapical cuttings respectively.

These tests demonstrated that high rooting frequencies could be achieved with ash cuttings supplied with auxin. Furthermore, it is possible to take at least two cuttings from one shoot i.e. an apical and a subapical cutting.

TABLE 3: EFFECT OF SHOOT APEX AND AUXIN TREATMENT ON ROOTING CAPACITY OF ASH CUTTINGS DERIVED FROM SEEDLINGS (16-30 CUTTINGS/TREATMENT).

Cutting type	*Auxin[1]*	*Rooting %*
Apex + 1st node	*None*	44
Apex + 1st node	*Seradix*	90
Apex + 1st node	*HRP*	75
Sub-apical 2nd node	*None*	34
Sub-apical 2nd node	*Seradix*	75
Sub-apical 2nd node	*HRP*	68
Apex + 2 nodes	*Seradix*	88
Apex + 2 nodes	*HRP*	72

[1]Auxin treatments applied to the cutting bases. Seradix: IBA 8000 ppm in talc, HRP: NAA 4000 ppm + 2% captan in powder.

TABLE 4: EFFECT OF AUXIN TYPE, CONCENTRATION AND AUXIN CARRIER WHEN APPLIED TO THE BASE OF CUTTINGS DERIVED FROM COPPICED ASH (25-35 CUTTINGS/TREATMENT).

Auxin treatment (mg L^{-1})	*Auxin carrier*	*Rooting %*
None	*Lanolin*	30
IBA (1000)	*Lanolin*	30
IBA (2000)	*Lanolin*	34
IBA (4000)	*Lanolin*	40
IBA (8000)	*Lanolin*	43
IBA (8000)	*Talc (Seradix)*	30
IBA (2000)	*Lanolin*	46
NAA (4000)	*Powder (HRP)*	37

Ash cuttings derived from apical cuttings and sub-apical cuttings were grown in 2l pots throughout the following season. Their height was measured at the end of the growing season. The mean height for plants derived from cuttings with a shoot apex was 79.38 cm ± 4.40 cm and for cuttings derived from sub-apical cuttings was 78.65 cm ± 6.56 cm. The differences were not statistically significant showing that there was no reduction in subsequent growth rates in cuttings which did not have a shoot apex.

The rooting capacity in terminal shoot cuttings derived from three clones of micropropagated plants of seedling ash was also tested. At the time of taking cuttings, the plants were pot bound. Rooting rate was clone dependant: giving 9% and 26% for each of two clones and 77% for clone 130/6 which also gave 81% rooting in cuttings from lateral shoots.

FIGURE 2: ROOT FORMATION IN ASH CUTTINGS
Roots emerging from the base of a Hassey tray after 12 weeks. Note more fibrous roots on cuttings taken from seedlings (left) and coarser roots on cuttings from hedged plants (right).

3.2 Rooting of ash and sycamore cuttings from coppiced hedges

Large-scale production of cuttings would be from hedges of elite clones. Annual trimming of the hedges would maintain them in a juvenile state. Rooting capacity was determined in cuttings from a hedge which was flail cut annually.

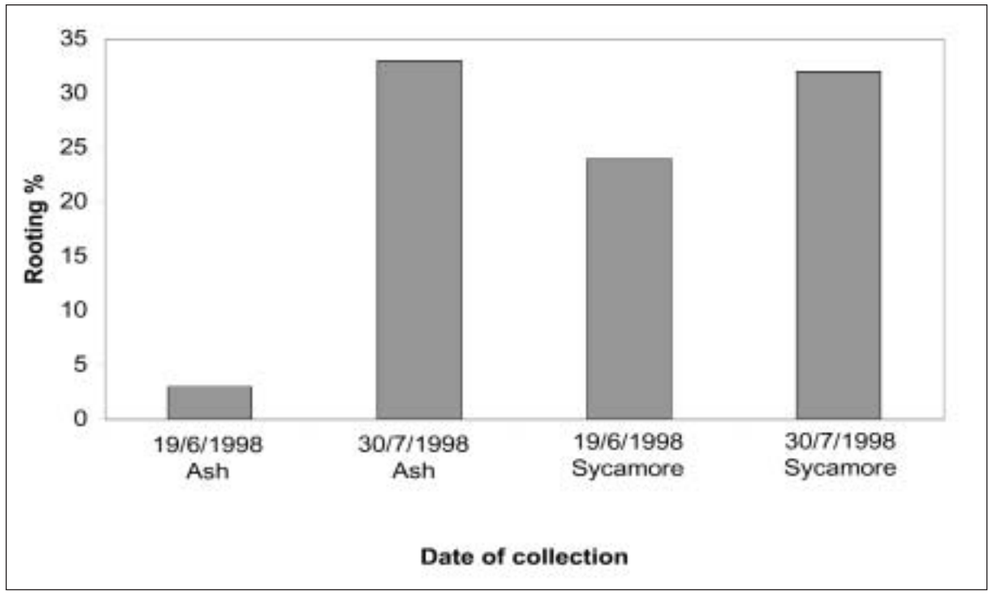

FIGURE 3 : EFFECT OF DATE OF COLLECTION ON ROOTING IN CUTTINGS FROM HEDGES OF ASH AND SYCAMORE. 86-121 cuttings/treatment, hedge flail cut in November annually, 15 different genotypes of each species.

Cuttings were collected at the end of August and auxin was applied as commercial product or in varying amounts in lanolin.

Results from ash (Table 4) showed less rooting in coppice shoots compared to seedlings. However, an increased rooting rate with increasing concentration of the auxin, IBA applied to the base of the cutting (maximum 46%) was observed. Roots on plants derived from hedging were coarser than those on cuttings taken from seedlings (Figure 2).

In the subsequent year, the influence of the stage of development of ash and sycamore shoots on their capacity for rooting in material from coppice was investigated (Figure 3). Cuttings of ash collected at the end of July gave a 10-fold increase in rooting percentage over those collected in mid-June. The later date of collection also yielded better rooting in sycamore (Figure 3).

3.3 Cuttings from grafted elite clones of ash

In the first experiments, cuttings were taken from grafted plants of eight elite clones of ash, dipped in Seradix and placed in enclosed mist. Only one clone rooted out of eight, with a rooting rate of 8%. Poor rooting in cuttings from mature trees is often attributed to physiological ageing and some treatments such as re-grafting new shoots may induce rejuvenation. In efforts to rejuvenate ash, scions from the original grafted plus trees were re-grafted on one, two, three or more successive occasions. The rooting capacity of cuttings, derived from plants that were grafted once, twice and four times, was recorded for six elite clones. Rooting capacity was generally low in all material (5 - 20%) with 2/6 clones failing to root. None of the clones rooted in cuttings taken from plants which had been re-grafted four times. In addition, cuttings from plants of the clone Jenkinstown 47, which had been re-grafted three and four times, and from the clone Athenry 4, which had been re-grafted four, five and six times, were tested. Cuttings from plants of each re-grafted stage of both of these clones failed to produce roots. These observations suggest that re-grafting had no effect on rooting capacity in cuttings.

3.4 Cuttings from grafted and self rooted elite clones of sycamore

A comparison was made between the rooting capacity of several sycamore clones using cuttings derived either from grafted plants or from plants which were self rooted. A mean rooting rate of 24% was obtained in cuttings from grafted plants. One clone failed to root out of eight tested (Table 5). In contrast, a mean rooting rate of 49% was obtained in cuttings from self-rooted clones and all

clones rooted. These observations suggest that self-rooted mother plants can be a source of cuttings with an improved capacity for rooting. Lower rooting rates in cuttings from grafted plants may be due to a greater physiological ageing in grafted plants or graft/scion incompatibilities.

3.5 Micropropagation of sycamore and ash

Sycamore clones were established *in vitro* using buds from grafted elite clones. Buds were cultured on QRC basal medium without growth regulators, containing 3 g L^{-1} charcoal (Table 6). High levels of bud sterility were obtained; results are summarised in Figure 4. Sycamore cultures failed to respond to applied cytokinins as other species. The establishment of an efficient micropropagation system was not possible. However, since sycamore can be propagated successfully by cuttings, it is proposed to use cuttings rather than micropropagated plants as the preferred method of providing plants for field testing.

There is little practical experience of clonal propagation of *Fraxinus* species. Micropropagation protocols have been published for juvenile material (Hammatt and Ridout 1992, Kim *et al.* 1997). For mature ash, a single clone was established in micropropagation by Hammatt (1994) and another by Pierik (1999). In this study, the hormone regime of Kim *et al.* (1997) was used to initiate and establish viable cultures from a range of ash clones.

Buds were collected from 40 selected trees in spring when bud flushing had just started (Table 7). Some trees provided over 30 buds for initiation of shoot cultures, others were small and less than 10 buds per clone were available. Surface sterilisation was satisfactory; 90% of the clones were free from surface contamination after 4 weeks. Out of 845 buds sterilised, 522 (62%) were free of fungal or bacterial contamination.

The viability of sterile buds from each of the 40 clones was tested over successive periods of culturing (Table 8). Buds were cultured on M9 medium. At the end of the second culture period, 40% of clones were necrotic, leaving 60% still viable. Thereafter, most clones survived for two further culture periods but by the sixth culture period only 20% of clones remained viable. All five clones which survived to the seventh culture period (12.5% of original amount) remained viable thereafter and became established as shoot-producing cultures.

The high mortality rate of ash buds during the establishment phase of shoot cultures has been attributed to the physiological maturity of the donor tissues. Successive grafting of new growth from grafted plants has been reported to induce rejuvenation in the grafted scion material. Over several years a re-grafting programme was carried out with selected clones of ash. The capacity of buds from some re-grafted clones to become established *in vitro* as viable cultures was observed.

TABLE 5: ROOTING CAPACITY IN CUTTINGS DERIVED FROM GRAFTED PLANTS OF ELITE CLONES OF SYCAMORE AND IN CUTTINGS COLLECTED FROM CUTTING-DERIVED PLANTS (SELF ROOTED).

Clone	*Cuttings from grafted plants*	*Cuttings from self-rooted plants*
	Rooting/clone %	
Gorey 60	n.t[1]	81.8
Piltown 34	n.t.	75.0
Bram 220-8	41.6	73.3
Bram 223-4	n.t.	52.6
Piltown 40	11.1	30.0
Camolin 81	n.t.	16.6
Durrow 79	n.t.	13.3
Durrow 75	27.0	n.t.
Athenry 1	30.0	n.t.
Athenry 2	21.4	n.t.
Athenry 6	25.0	n.t.
Knocktopher 45	36.6	n.t.
Durrow 66	0.0	n.t.
Mean rooting	24%	49%

[1]n.t. = not tested

TABLE 6: COMPOSITION OF QRC MEDIUM.

Chemical	*Final concentration mg L^{-1}*
NH_4NO_3	400.00
$Ca(NO_3)_2\ 4H_2O$	556.00
K_2SO_4	990.00
$CaCl_2\ 2H_2O$	96.00
Solution 4[1]	10.00(ml)
H_3BO_3	6.20
$Na_2MoO_4\ 2H_2O$	0.25
$MgSO_4\ 7H_2O$	370.00
$MnSO_4\ 7H_20$	22.30
$ZnSO_4\ 7H_2O$	8.60
$CuSO_4\ 5H_2O$	0.25
Thiamine HCl	0.25
Inositol	50.00
Sequestrene Fe 330[2]	40.00

[1] Solution 4 i.e. Ammonium phosphate instead of potassium phosphate
Preparation Steps for Solution 4
a. 0.053g $(NH_4)_2HPO_4$ in 50 ml H_2O
b. 0.573 g $NH_4H_2PO_4$ in 50 ml H_2O = (0.1 M)
c. Take 45 ml solution No. 2 and add to 50 ml solution No. 1
d. Adjust pH to 5.8 using No. 2 solution = Solution 4
e. Bring to final volume 100 ml
f. Store in freezer
g. Use 10 ml of Solution 4 per 1.0 L medium
[2] Sequestrene Fe 330 (Geigy) instead of Fe-EDTA

Cultures from clone Thomastown 70 were successfully established with buds collected in May 1998, from field-grown plants grafted once. In contrast, there was a failure to establish viable cultures from this same clone with buds collected at the same time in May 1998, from glasshouse grown plants, which had been re-grafted three times. Similarly, with clones Jenkinstown 48 and Athenry 7; viable cultures were not established using buds from plants which had been re-grafted 3-5 times. These observations suggest that re-grafting (onto 2-year-old rootstocks) did not facilitate the establishment of viable cultures from several ash clones. Similarly, rooting was not improved in cuttings from regrafted plants. It may be concluded that re-grafting as performed here, was not effective in rejuvenating trees. Perhaps using more juvenile rootstocks such as 1-year-old rootstocks (rather than conventional 2-year-old), and larger pots, to give a greater development of roots, may be a more effective method to rejuvenate ash.

Care was taken not to sub-divide the shoot cultures excessively during the first five subcultures since this caused necrosis of tissues and death of the shoot explants. Subculturing consisted of bisecting any elongated shoots resulting in two types of explant, one with an apical bud which continued to grow slowly and the other with axillary buds, only one of which resumed growth. For both types of explant, the stem became highly lignified. In the first few subculture periods, the propagation rate was less than one for most clones. The highest propagation rates were in the August - September period. Clone Monasterevin 72 was the most productive giving a maximum of four explants per original explant cultured.

The propagation rates achieved in these first trials were too low for large-scale production of selected lines so an alternative method for sub-dividing the shoots in established cultures was tested. Shoots were dissected to give three types of explant for culturing:

(i) apical buds;
(ii) individual nodes, consisting of two opposite buds (with leaves detached); and
(iii) multi nodal stems with basal callus attached.

In this way, a normal shoot could be sub-divided into three or four explants. The capacity of single nodes to produce shoots from axillary buds is summarised for three clones in Table 9.

After 4 weeks of culturing, 71-92% of nodes produced a shoot, depending on the clone and medium used. Clone 8x was most responsive with 70-96% of nodes producing a shoot. The higher concentration of the cytokinin (TDZ), in medium

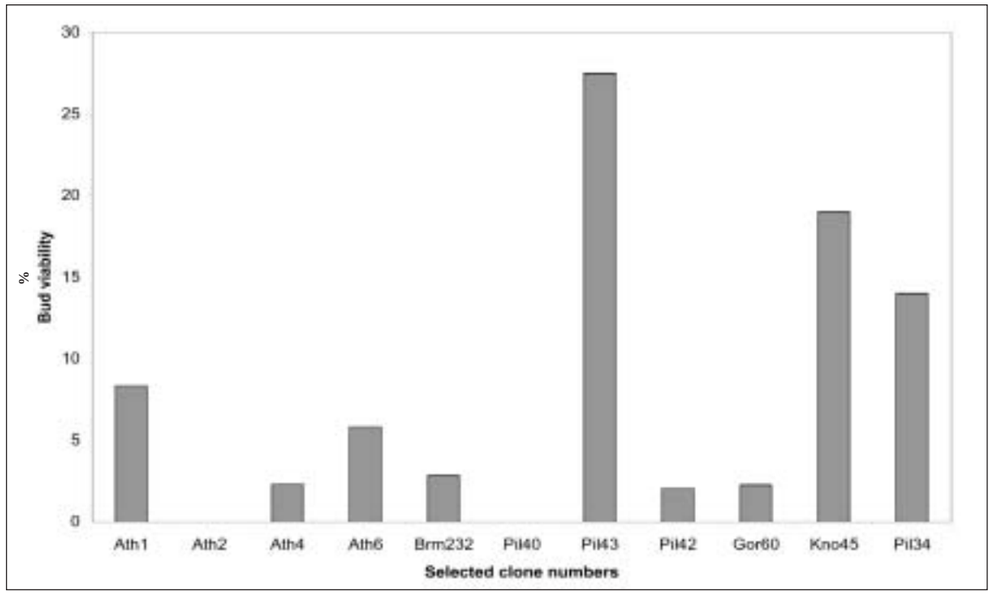

FIGURE 4 : VIABILITY OF SELECTED CLONES OF SYCAMORE AFTER THREE SUBCULTURES ON QRC MEDIUM

M9, favoured shoot development from single nodes. Nodes of this clone which were recultured twice, without dissection, to fresh media resulted in final shoot lengths of 22, 25, 23 and 19 mm on media M9, N9, N13, and N14 respectively. In addition, these cultures also produced secondary shoots in 47% of cultures on M9, 17% on N9, 31% on N13 and 6% on N14. From this and other experiments, the hormone regime of M9 was adopted as the standard medium and its effects tested on shoot production in four juvenile seedling clones of ash. Nodes and apices were used as the explants and micropropagation rates of 4.1, 2.4, 5.2, and 3.4 were obtained for each genotype respectively.

The appearance of ash shoots which were cultured for several months on the standard medium M9 became glassy, water-soaked and unhealthy (vitrification). This abnormality is generally caused by excessive cytokinins in the medium and can lead to the loss of cultures. Therefore, the

TABLE 7: DESIGNATION OF 40 ASH PLUS TREES[1] FROM WHICH BUDS WERE INITIATED INTO MICRO PROPAGATION IN 1998.

Forest	*Designated number*	*Forest*	*Designated number*	*Forest*	*Designated number*
Athenry	6	*Castlecomer*	47	*Monasterevin*	74
Athenry	7	*Cong*	2	*Monasterevin*	77
Athenry	8	*Donadea*	23	*Portlaoise*	78
Athenry	38	*Dundrum*	80	*Portlaoise*	79
Athenry	53	*Dundrum*	81	*Portlaoise*	85
Athy	36	*Durrow*	54	*Roscrea*	1
Athy	37	*Jenkinstown*	48	*Shelton*	10
Athy	51	*Jenkinstown*	49	*Shelton*	11
Athy	52	*Knocktopher*	64	*Shelton*	12
Avonmore	82	*Knocktopher*	75	*Shillelagh*	62
Castlecomer	40	*Knocktopher*	76	*Stradbally*	84
Castlecomer	41	*Monasterevin*	72	*Thomastown*	70
Castlecomer	46	*Monasterevin*	73	*Virginia*	83

[1]Each plus tree has been grafted.

TABLE 8: INITIATION OF CULTURES OF 40 SELECTED MATURE ASH IN 1998, VIABILITY OF BUDS AT THE END OF EACH SUCCESSIVE PERIOD OF CULTURING.

Culture period	*No. clones viable*	*Clones viable %*	*Clones lost %*	*Number of viable clones at each culture period*
1st	36	90.0	10.0	40
2nd	24	60.0	30.0	29
3rd	15	37.5	22.5	20
4th	15	37.5	0.0	20
5th	9	22.5	15.0	9
6th	8	20.0	2.5	9
7th [1]	5	12.5	7.5	5

[1]Clones which were viable after seven subcultures remained viable and became established as shoot producing cultures. M9 medium used throughout i.e. MS medium + TDZ 1.1 mg L^{-1}, BA 5.0 mg L^{-1}, IBA 0.2 mg L^{-1}. The five viable clones were Athenry 8 and 6, Thomastown 70, Monasterevin 72 and Shelton 10.

shoot cultures were transferred to hormone-free medium (QRC) containing 3.0 g L^{-1} activated charcoal. On this medium, the explants reverted to produce healthy shoots, which could be further propagated. The yields of explants obtained on QRC and M9, from three types of original explant (apex, single node and multiple node) for two clones are summarised. Although the propagation rate declined by culturing explants without growth regulators (Table 10), the new shoots produced were healthy in appearance and often rooted spontaneously. Spontaneous rooting may be indicative of rejuvenation. To maintain the health of cultures and consistent rates of propagation the material was routinely cultured onto media containing growth regulators (M9) followed by a period without growth regulators (QRC).

The rooting capacity of six seedling clones *in vitro* and the effects of three concentrations of IBA was tested (Table 11). One-hundred percent rooting was achieved for three clones; the optimal concentration of IBA varied for each clone. For the mature clone 8x, the initial rooting trial gave

TABLE 9: EFFECT OF FOUR MEDIA ON PRODUCTION OF SHOOTS FROM SINGLE NODE EXPLANTS OF THREE MATURE CLONES OF ASH *IN VITRO.*

Medium[1]	*% of nodes producing shoots in clones (mean shoot length)*					
	F5		*JK47*		*8x*	
	%	*mm*	*%*	*mm*	*%*	*mm*
M9	82	2.7	71	5.7	92	2.3
N9	29	1.1	71	2.9	96	8.8
N13	39	1.3	57	1.7	96	10.2
N14	50	3.2	-		70	5.8

[1]M9: TDZ 1.1 mg L^{-1}, BA, 5.0 mg L^{-1}
N9: TDZ 0.11 mg L^{-1}, PBA, 2.5 mg L^{-1}
N13: TDZ 0.11 mg L^{-1}
N14: TDZ 1.1 mg L^{-1}, PBA, 2.5 mg L^{-1}

TABLE 10: EFFECT OF MEDIA ON PRODUCTION OF EXPLANTS BY APICES, SINGLE NODES AND MULTI-NODES IN TWO ELITE CLONES OF ASH CULTURES.

Clone	*Medium*[1]	*Mean yield of explants obtained per:*		
		shoot apex	*single node*	*multi node*
Monasterevin 72	*QRC*	1.2	1.2	2.6
	M9	3.2	3.9	2.6
Jenkinstown 49	*QRC*	1.0	1.0	2.7
	M9	1.0	-	1.3

[1]M9: MS medium + TDZ 1.1 mg L^{-1}, BA 5.0 mg L^{-1}, IBA 0.2 mg L^{-1}.
QRC: Basal medium, no hormones, 3% charcoal (Table 10).

TABLE 11: ROOTING IN SIX DIFFERENT GENOTYPES OF ASH AND THE EFFECTS OF INDOLE BUTYRIC ACID (IBA) MS MEDIUM ½₀ STRENGTH, SUCROSE 3%.

IBA	*Seedling genotype*					
	1	2	3	4	5	6
mg L⁻¹	*Rooting per clone %*					
0	83	12	-	89	77	-
1.0	-	-	91	100	-	89
5.0	50	33	100	100	100	69
10.0	-	-	-	100	85	-

58% rooting with 5.0 mg L^{-1} IBA and 40% in the absence of IBA. Root number per cutting was 2.3 with IBA and 1.3 in the absence of IBA. These studies indicated that rooting was stimulated by IBA and rooting potential was high in seedling as well as in micropropagated mature clones of ash.

Using the micropropagation methods described above for the selected ash clones from mature trees, approximately 100 plants were produced which were transferred to the greenhouse for further field testing (Figure 5).

FIGURE 5: MICROPROPAGATED PLANTS OF SELECTED MATURE ASH TREES IN THE GREENHOUSE AT THE END OF THE FIRST GROWING SEASON.

4. CONCLUSIONS

1. Vegetative propagation of ash and sycamore is a feasible approach to use as one element in the genetic improvement of these important broadleaves.

2. Elite trees were selected among all mature trees in our native populations; grafting resulted in the conservation of all elite ash and 73% of elite sycamore.

3. Rooting rates of up to 90% were obtained for ash cuttings that were collected from juvenile seedlings, indicating a high rooting potential among many genotypes.

4. The propagation of mature, elite ash trees by cuttings is still not optimal using material from grafted plants. However, rooting of cuttings collected from micropropagated plants is more promising.

5. Propagation of elite ash trees by micropropagation was possible for 12.5% of the clones tested. Micropropagation allows for the large-scale production of plants and may provide rejuvenated plants with a high rooting capacity in cuttings from micropropagated donor plants.

6. The efficiency in micropropagation of ash was improved by using single buds and nodes as the cultured explants rather than whole or bisected shoots.

7. We established shoot cultures from eleven elite sycamore clones. However, micropropagation of sycamore requires more development since the propagation rates obtained were too low for practical use.

8. Collecting cuttings of ash and sycamore from elite plants which are maintained as hedges may provide a practical way of maintaining elite plants which provide a succession of cuttings with a high capacity for rooting.

9. Serial grafting (re-grafting) of ash was not a successful method of rejuvenating plants. There was no increase in the capacity of cuttings to root or of buds to become established as viable shoot cultures, in material which was re-grafted many times.

10. Sycamore cuttings showed a high rooting capacity among 13 clones tested and 25% of cuttings from grafted plants produced roots. When cuttings were taken from these rooted plants the rooting percentage increased to 49%. This indicates that some rejuvenation, and recovery of rooting capacity may have taken place in sycamore. This would offer the potential to develop large-scale vegetative propagation of sycamore via cuttings.

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PHOTOAUTOTROPHIC MICROPROPAGATION OF ASH AND SYCAMORE

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SUMMARY

Ash (*Fraxinus excelsior* L.) and sycamore (*Acer pseudoplatanus* L.) are important forest tree species in Ireland. Methods of vegetative propagation should be available if the genetic potential available of indigenous elite populations is to be exploited in the short to medium term.

Two methods of vegetative propagation of these species have been studied previously, production by conventional cuttings and by conventional micropropagation. Use of conventional cuttings is possible in the case of sycamore but it is not an efficient system and the economic costs are deemed too high for the level of gain likely to be achieved. In the case of ash it is simply not possible to root conventional cuttings at anything other than very low, uneconomic rates. Micropropagation has long been suggested as a potential alternative to traditional methods of vegetative propagation. Unfortunately, in spite of many years of research effort around the globe, this approach has not been successful in developing a consistent methodology at the laboratory level, much less a fully commercial system.

In the course of this study, much of the published work on conventional micropropagation of these species was repeated for comparison purposes and many of the problems experienced by other researchers were confirmed. Photoautotrophic micropropagation methods were investigated as a possible way of overcoming the major hurdles experienced with conventional micropropagation. Procedures were developed for the direct establishment of material from mature sycamore under photoautotrophic conditions. This was best achieved using semi-hardwood explants in the summer and autumn months. This material was then used in a microcoppice or microstoolbed to produce a supply of softwood microcuttings for rooting, weaning and establishment. In the case of ash, direct establishment of microstoolbeds was not possible but bud break and stem elongation from material excised from mature trees was achieved under photoautotrophic conditions at any time of the year. The shoots from these cultures were then used to establish microstoolbeds. With successive harvesting of microcuttings from these cultures the percentage of successful rooting increased to 90%, offering for the first time a potentially commercial method for the micropropagation of ash selected from mature trees. In the case of both species, it has now been shown that there is a pathway for efficient vegetative propagation suitable for transfer to the industry.

The level of regeneration, the numbers of microcuttings obtained from the microstoolbeds, the longevity of the cultures, the possible manipulation by plant growth regulators and other factors such as the size, number and quality of the cuttings all require further work for the full potential of the technology to be elucidated and before a full economic analysis of the system can be concluded.

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1. INTRODUCTION

The broadleaved species of interest to Irish forestry are all outbreeders. The ability to clonally propagate them in order to multiply individuals or full-sib families from breeding programmes that exhibit advantageous characteristics may offer strategic advantages to the industry.

The usual approach to clonal propagation is by either *in vivo* cuttings or conventional micropropagation. The relative merits of these methods are well documented. Propagation by rooted cuttings is slow, seasonal and at best exhibits variable success rates. Conventional micropropagation is a technique that is applied to high value ornamental and plantation crops. The problems and costs associated with conventional micropropagation are such that it is not now, nor is it ever likely to be, a cost effective technique for the production of large volumes of forestry planting material, unless a large commercial advantage is to be gained from the use of elite planting material.

There are two alternatives to conventional micropropagation that offer a potential solution to some of the problems associated with that technique. These are somatic embryogenesis and photoautotrophic micropropagation. Somatic embryogenesis is considered by many authorities to be the long-term future for clonal propagation of forestry species.

Photoautotrophic micropropagation is a method of micropropagation where the sucrose is removed from the tissue culture medium and the plant material is placed in conditions of enhanced light level and elevated carbon dioxide concentrations to encourage the material to photosynthesise. The presence of sucrose, leading to a high contamination risk, physiological problems and the use of small containers is now recognised as a major factor associated with production problems and high production costs in conventional micropropagation.

The objective of this project was to examine the technique of photoautotrophic micropropagation and its application to ash and sycamore and develop a cost effective technique that would be of application in Irish forestry, either in the production of clonally propagated material for plantation establishment or as a tool in the development of the wider scale use of adapted germplasm in Ireland.

Hammatt (1996) reported that an immediate and key objective of ash biotechnology is to develop means to clonally propagate selected trees, thus enabling rapid genetic gain to be achieved with this species, which has been studied little genetically, and has a long breeding cycle, flowering when 15-20 years old. Given difficulties with the rooting of cuttings, successful propagation *in vitro* could be used to generate direct planting stock for both clonal trials and commercial woodland.

2. MATERIALS AND METHODS

Various source materials were used to establish cultures of both sycamore and ash. These included seeds, dormant buds from both juvenile and mature sources, shoot cuttings from both juvenile and mature sources and epicormic shoots.

2.1 Medium preparation and use

Throughout this study a variety of plant tissue culture media were used. These included MS medium (Murashige and Skoog 1962), Woody Plant medium (WPM) (Lloyd and McCown 1980), DKW medium (Driver and Kuniyuki 1984) and Enshi medium (Hori 1966). All of the media were made up from analar grade reagents maintained as stock solutions in a cold room (4 ˚C) or freezer (-20 ˚C) and were autoclaved at 121 ˚C, 1.03 Nm^{-2} prior to use.

A variety of substrate were used. Agar (Sigma Type IV) was used as the primary gelling agent. In addition gelrite, vermiculite, perlite and a variety of horticultural polyurethane foam substrates were used.

2.2 Growth room conditions

Cultures were placed in conventional growth rooms at a temperature of 20 ± 2 ˚C, with a 16 hr-photoperiod and a photosynthetic photon flux density (PPFD) of 30 µmol $s^{-1}m^{-2}$. For photoautotrophic conditions the cultures were placed in a growth room with either 90 or 130 µmol $s^{-1}m^{-2}$, with a 16 hr-photoperiod, carbon dioxide enrichment (CDE) external to the culture vessel of 3000 ppm and a temperature of 23 ± 2 ˚C.

2.3 Establishment and culture of ash seed *in vitro*

Fresh or dry seeds were immersed in 1% sodium hypochlorite (w/v) with a few drops of 0.01% Tween for 20 for 30 minutes. They then were rinsed five times with sterile distilled water. The apical third of seeds (opposite radicle end) was aseptically excised prior to inoculation in test tubes containing shoot induction medium (half strength MS + 3% sucrose, 0.7% agar, pH 5.7).

2.4 Establishment and culture of dry seed of sycamore *in vitro*

Sycamore seeds (samaras) were collected from a single tree on the Belfield campus of University College Dublin in October 1995. Dry seeds were picked from the ground and stored until used. Samaras were washed in running tap water and soaked in 5% Domestos overnight. After rinsing several times in distilled water, the pericarp was removed and the seeds were soaked in 1.0 g L^{-1} Benlate for 30 minutes and rinsed thoroughly with distilled water. Seeds were transferred to 20% calcium hypochlorite solution for 30 minutes and washed 3 times in sterile distilled water. Seeds were then placed in sterile petri dishes on autoclaved filter paper moistened with sterile distilled water and incubated for 48 hrs at 21 ± 3 ˚C in the dark. The testa of each seed was then removed and each was sterilised by immersion in 85% ethanol for 30 seconds, followed by 15 minutes in 7% calcium hypochlorite. Seeds were rinsed five times in sterile distilled water and cultured in half strength MS medium with 6.0 g L^{-1} agar, 3% sucrose, and pH 5.6 - 5.8.

2.5 Establishment and culture of fresh seed of sycamore and ash *in vitro* under enriched carbon dioxide conditions

Fresh seeds of ash and sycamore were collected in the middle of September 1997. Samaras were washed for one hour under running tap water and sterilised with 5% Domestos for 30 minutes. Seeds were extracted from the samaras and their pericarps were removed. The testa of each sycamore seed was removed and the seeds were soaked in 0.1% Captan for 30 minutes, then transferred to 7% calcium hypochlorite for 15 minutes. After washing five times with sterile distilled water, sycamore seeds were cultured in test tubes containing half strength MS sugar free medium with 0.6% agar, pH 5.7.

With ash it was necessary to aseptically excise one third of each seed opposite the radicular end prior to inoculation in order to facilitate gas diffusion and cotyledon emergence. The seeds were cultured in test tubes containing half strength sugar-free MS medium with 0.6% agar, pH 5.7. All cultures were held at 20-24 ˚C with a 16 hr-photoperiod and a light intensity of 130 µmol $s^{-1}m^{-2}$.

2.6 Direct establishment of sycamore softwood and semi-hardwood cuttings under enriched carbon dioxide conditions

Cuttings were collected from sycamore trees on the Belfield campus in July 1997. Cuttings were washed for 30-60 minutes under running tap water, soaked for 30 minutes in 1.0 g L^{-1} Benlate, followed by surface sterilisation in 5% calcium hypochlorite with a few drops of Tween 20 and then rinsed five times with sterile distilled water. Each cutting with two nodes was treated with either rooting powders or a dip in an auxin solution. Cuttings were cultured immediately in sterilised Magenta vessels with vented GA-7 lids containing sugar and growth regulator (GR) free liquid Enshi medium. Vermiculite, hortifoam and vermiculite plus gelrite were used as substrates. Cultures were transferred into CDE (3000 ppm CO_2) conditions at 21-24 ˚C with a 16 hr-photoperiod and a light intensity of 90 µmol $s^{-1}m^{-2}$.

2.7 Production of epicormic shoots from mature ash and sycamore

Trees approximately 20 years old, growing on the Belfield campus, were felled and the trunks cut into logs about 40 cm long. Logs from each species were soaked for 2 hours in 1 mg L^{-1} GA_3, BAP, or TDZ and left standing in 50 cm pots of Shamrock potting compost (Bord na Mona, Ireland) and covered with clear plastic in a heated glasshouse. The number of epicormic shoots produced was counted after two months.

2.8 Experimental designs and statistical analyses

The experimental designs used are outlined in the results section but usually consisted either of a completely randomised block design, or a factorial randomised block design where two or more treatments were being examined at a number of different levels. The data obtained were analysed using the Statistical Analysis System using a general linear model (SAS 1985).

3. EXPERIMENTAL WORK: RESULTS AND DISCUSSION

3.1 Establishment of *in vitro* cultures

A number of experiments were performed to establish selected material in *in vitro* culture. In an ideal situation, the ability to select material from semi-mature or mature trees and multiply it *in vitro* would be desirable. The literature indicates that this is practically impossible without first inducing a phase change back to juvenile characteristics. In order to ensure a supply of material for further experimentation, the approach taken here was to use the traditional route of initiating cultures from germinating seeds under mixotrophic conditions. However, more novel approaches such as germinating seeds under photoautotrophic conditions, the use of juvenile and mature shoot cuttings containing dormant buds, the use of softwood and semi-hardwood cuttings as explants and finally the induction of epicormic shoots from the trunks of mature trees for use as explants were also attempted.

3.1.1 Seed germination under mixotrophic conditions

Ash and sycamore seeds were treated as previously described (Section 2.3 and 2.4) and placed on 1/2 MS medium containing 3% sucrose solidified with agar. These were placed in a growth room under the standard mixotrophic conditions. In more than 95% of the cultures, fungal and bacterial contaminants quickly overwhelmed the cultures and the material was lost. This was in spite of repeated attempts with various seed lots of both fresh and aged seed at different times of the year.

3.1.2 Seed germination under photoautotrophic conditions

Seeds were treated as described previously (Section 2.5) and placed on 1/2 MS sugar-free medium solidified with agar and cultured under photoautotrophic conditions. With sycamore, this resulted in 100% germination and no visible fungal contamination of the cultures. Bacterial contaminants were visibly present in approximately 5% of cultures. The seeds germinated approximately one week after commencement of *in vitro* culture; 15-20 cm growth was recorded after four weeks.

Germination of ash seed was much lower, 22%, possibly as a result of seed dormancy due to the under-development of the embryo at the time of fruit collection. This is well documented in the literature (Boner 1974, Young and Young 1992) and can be overcome by after-ripening the fruit and/or by stratification. Such techniques were not attempted within this study.

Culture of seeds on sugar-free medium under CDE conditions provides for fast *in vitro* seedling establishment with a high percentage of seed germination (particularly in the case of sycamore) and virtually no visual contamination by saprophytic organisms. This is a valuable option to establish *in vitro* seedling cultures from elite materials, particularly where the supply of material is limited and the risk of loss of material through contaminating fungal and bacterial species is required to be minimised. Excellent germination and initial growth was achieved under these conditions and while there was little or no obvious contamination problems, the material could not be regarded as axenic.

3.1.3 Direct establishment of sycamore semi-hardwood cuttings under autotrophic conditions

A trial was set up examining the effect of different hormone rooting treatments and substrates on rooting ability. The nutrient medium used was Enshi. Hormone rooting treatments consisted of placing the basal end of the cutting into either a commercial rooting powder, or IBA solutions of 2500, 5000, or 10000 ppm. The control consisted of a dip in distilled water. Thirty replicate cuttings were used for each hormone/substrate combination.

The type of substrate, concentration of IBA and their interactions were all significant. The hortifoam substrate yielded a significantly higher percentage rooting than vermiculite, or vermiculite plus gelrite. Rooting powder was the most effective hormone treatment for rooting of sycamore semi-hardwood cuttings (Table 1).

There was a significant interaction between substrate and hormone. The maximum rooting occurred in the cuttings, which were planted in hortifoam and treated with rooting powder (Table 2).

In a separate trial the inclusion of IBA in the medium (in contrast to a dip of the basal end of the cutting in IBA) at a range of concentrations, was

TABLE 1: EFFECT OF DIFFERENT CONCENTRATIONS OF IBA, ROOTING POWDER AND SUBSTRATE TYPE ON ROOTING OF SYCAMORE SEMI-HARDWOOD CUTTINGS.

IBA concentration (ppm)	*Rooting %*	*Substrate*	*Rooting %*
0	18c[1]	*Hortifoam*	41a
2500	11c	*Vermiculite*	5c
5000	11c	*Vermiculite + gelrite*	21b
10000	27b		
Rooting powder	47a		

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$).

used in an attempt to root sycamore semi-hardwood cuttings. In addition, a cold pre-treatment (4 ˚C in the dark) was used in an attempt to improve the percentage of cuttings rooted. In this case, vermiculite was significantly superior to hortifoam in the control treatment. IBA at 1.0 mg L^{-1} significantly increased the percentage rooting in the case of hortifoam, but there was no significant increase when vermiculite was used as a substrate and at this level of IBA there were no significant differences between the substrates (Table 3).

Cold pre-treatment for two days prior to inoculation did not significantly increase the percentage of cuttings rooted. Longer pre-treatment led to higher levels of necrosis and death of the cultures. Nodal cuttings, where they survived, produced callus at the basipetal end of the cuttings but the level of rooting was poor.

3.1.4 Production and culture of epicormic shoots from mature ash and sycamore

Epicormic shoots from both ash and sycamore were produced, in the manner previously described (Section 2.7), from logs in September. For both species approximately five or six shoots up to 12 cm in length were produced within a two-month period, irrespective of hormone pre-treatment used. Attempts to introduce these shoots to culture resulted in a large percentage of the material becoming contaminated, primarily with algal growth. This arose mainly due to the use of non-sterilised peat based compost for standing the shoots in and the use of polythene bags to maintain humidity.

TABLE 2: EFFECT OF SUBSTRATE TYPE AND PGR ON ROOTING OF SYCAMORE SEMI-HARDWOOD CUTTINGS.

Substrate	*Hormone ppm*	*Rooting %*
Hortifoam	0	40[3]
Hortifoam	2500	33[3]
Hortifoam	50000	20[1]
Hortifoam	10000	27[2]
Hortifoam	Rooting powder	87[3]
Vermiculite	0	0
Vermiculite	2500	0
Vermiculite	5000	19
Vermiculite	10000	0
Vermiculite	Rooting powder	13
Vermiculite + gelrite	0	13
Vermiculite + gelrite	2500	47[3]
Vermiculite + gelrite	5000	0
Vermiculite + gelrite	10000	7
Vermiculite + gelrite	Rooting powder	40[3]

[1]$p \leq 0.05$ [2]$p \leq 0.001$ [3]$p \leq 0.0001$

3.1.5 Disinfection procedures

One of the major problems in micropropagation of woody species is the introduction of material into culture and removal of contaminating fungi and bacteria from the explants. This was reinforced by the experiences during this project. While the absence of sucrose in the medium meant that contaminating microorganisms did not have readily utilisable substrate to grow on, it was still desirable and represents good laboratory practice to disinfect the starting material. In this trial, the effect of various disinfection procedures in removing contaminating microorganisms from sycamore semi-hardwood nodal explants was examined.

Sycamore semi-hardwood cuttings were taken from mature trees on the Belfield campus. Explants consisting of two nodes were treated as described in Section 2.7. After the initial tap water wash, material was either treated with Benlate or left untreated. Explants were then subjected to disinfection using one of four disinfectants at four different concentrations. After further washing in sterile distilled water the explants were placed on half strength MS medium containing sucrose (30 g L^{-1}) and solidified with agar. The number of contaminated nodes was counted and the type of contaminant assessed; the number of damaged or necrotic nodes and the number of nodes exhibiting a halo of dark phenolic substances in the agar were recorded.

In all cases, where explants were pre-washed in Benlate, the level of contamination was significantly decreased. Domestos was less effective than other disinfectants in reducing the level of fungal contamination (Table 4). None of the disinfectant treatments used was effective against bacterial contaminants and there were no significant differences in that regard. This presumably reflects the fact that the bacterial contaminants were either endogenous in origin or were protected in some manner on the epidermis of the explant during the disinfection procedure. Calcium hypochlorite caused significantly less damage on sycamore explants and there were significant differences between calcium hypochlorite and the other disinfectants. Surface sterilisation with calcium hypochlorite resulted in less phenolic compounds being secreted in the medium as measured by visual assessment of the cultures (Table 4).

3.1.6 General discussion on the establishment phase

Establishment of material from woody plants in *in vitro* culture is always problematic. One of the major barriers to success is the large exogenous and endogenous microflora that quickly over-run the medium and destroy the culture. Another major problem is the presence of phenolic compounds that move out of the explant into the medium preventing or stunting growth and often contributing to the death of the explant. Trials conducted in the course of this study have confirmed these problems, particularly when material is placed under mixotrophic conditions, with sucrose present in the medium.

Progress in tissue culture of ash has been made only relative recently, and all of the reports have been with juvenile material (Chalupa 1983 and 1993, Hammatt and Ridout 1992, Hammatt 1994). Hammatt and Ridout (1992) used seedlings because their early work with mature ash had failed due to contaminating microorganisms in the

TABLE 3: EFFECT OF DIFFERENT CONCENTRATIONS OF IBA AND TYPE OF SUBSTRATE ON ROOTING OF SYCAMORE SEMI-HARDWOOD CUTTINGS (WITHOUT COLD TREATMENT).

Substrate	*IBA mg L^{-1}*	*Rooting (0 day) %*
Hortifoam	0.00	13
Hortifoam	0.25	19
Hortifoam	0.50	6
Hortifoam	1.00	31[2]
Vermiculite	0.00	25[2]
Vermiculite	0.25	31[2]
Vermiculite	0.50	44[2]
Vermiculite	1.00	31[2]

[1]$p \leq 0.05$ [2]$p \leq 0.001$ [3]$p \leq 0.0001$

TABLE 4: EFFECT OF DISINFECTANT TYPE ON FUNGAL CONTAMINATION, OCCURRENCE OF NON-DAMAGED EXPLANTS AND PRODUCTION OF PHENOLIC COMPOUNDS ON SYCAMORE EXPLANTS.

Type of disinfectant	*Fungal contamination*	*Phenolic compounds*	*Non-damaged explants*
		%	
Domestos	20a[1]	53ab	24b
Calcium hypochlorite	0b	33c	39a
Sodium hypochlorite	0b	48b	27b
Milton	0b	61a	27b

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$).

cultures. Conventional disinfection methods were unsuccessful, since plant tissues were damaged by long exposure time to sterilants, which was necessary to remove exogenous contaminants. Endogenous bacteria and fungi (e.g. *Rhizoctonia, Alternaria and Stilbella*) could not be eliminated by surface sterilisation of plant material.

Hammatt (1996) reported that induction of old ash material from mature ash trees into culture was hampered by difficulties in surface sterilisation of the initial explants. In the trials reported here, various combinations of treatments failed to produce uncontaminated cultures. In one experiment, only two 'clean' cultures were obtained from 120 initial explants of one clone. Even then, contaminating *Bacillus* appeared from these after four months.

Part of the reason for the severe contamination may have been associated with the time of year in which the cuttings were taken (January-February). It is widely reported that shoots taken during the winter months become severely contaminated and disinfection is particularly difficult.

Selection of explant material in the proper physiological condition may help to ensure successful culture initiation. It is commonly accepted that actively growing shoot tips are the best explants to use for initiating shoot cultures (Kyte 1987). Shoot tips collected at various times during the spring growth flush, varied considerably in their ability to initiate proliferating shoot cultures. Experiments used flushed buds from dormant material in January and February and shoot tip cultures in April. Shoot tip cultures taken in April suffered from both bacterial contamination and the production of phenolic compounds, leading to explant death. By taking explants in May to June and modifying the disinfection procedure, the success rate was improved but without totally eliminating the problems.

The results of these experiments confirmed that pre-washing of plant materials with a fungicide is necessary. Surface sterilisation with calcium hypochlorite resulted in more clean explants with less damage and in addition production of phenolic compounds decreased in the culture medium.

3.2 Shoot growth and multiplication of ash and sycamore *in vitro*

Having established material from a variety of sources during the initial phases of the project, the next objective was to achieve multiplication of the material. The accepted best practice in the case of woody plant propagation is to avoid any callus production and to encourage the development of axillary buds. From the viewpoint of achieving multiplication, an option is to achieve extension growth of the shoot and to then subdivide the shoot into nodal cuttings. A better option from a multiplication point of view is to achieve multiple bud and shoot development within the axillary bud. Under mixotrophic conditions, this is achieved by including plant hormones in the medium at low concentrations. Experience with autotrophic micropropagation is that conditions that are successful in conventional micropropagation rarely translate directly to autotrophic micropropagation.

The experimental work in this section looked initially at conventional multiplication methods using juvenile material under mixotrophic conditions. This was then expanded to examine the effect of high light and carbon dioxide enrichment and finally the use of cuttings from mature trees under autotrophic conditions.

3.2.1 Effect of medium, BAP and activated charcoal on shoot growth and multiplication rate of ash and sycamore nodal explants

Shoots from *in vitro* grown seedlings of ash were removed from the tissue culture jars and divided into nodal segments, with two buds on each segment. Leaves subtending the buds were removed. These were then cultured on media in Wilsanco tubs using WPM and DKW media, with three different concentrations of BAP and in the presence or absence of activated charcoal. The experiment consisted of a factorial arrangement in a completely randomised design.

Activated charcoal, concentration of BAP and the interaction between BAP concentrations and activated charcoal were significant for shoot height, multiplication rate and callus formation at the base of nodal explants (Table 5). The interaction between media and activated charcoal was significant on shoot height. DKW was significantly better than WPM for shoot height of nodal explants. Minimum shoot multiplication and callus formation was in a medium containing 1.0 mg L^{-1} BAP which was significantly different from the higher concentrations of 5.0 and 2.5 mg L^{-1} BAP. The shoot height of nodal explants was maximum in a medium with 2.5 mg L^{-1} BAP, median with 1.0 mg L^{-1} BAP and was least at 5.0 mg L^{-1} BAP and differences between the three concentrations were significant (Table 5).

Medium without activated charcoal significantly increased shoot multiplication, shoot height and callus formation at the base of cuttings (Table 6).

In general, DKW medium was better than WPM medium for shoot growth and elongation while the multiplication rate was not significantly affected by medium. Maximum shoot multiplication (2.71 ± 1.25) and shoot growth (3.01 ± 1.04 cm) was achieved on DKW medium containing 2.5 mg L^{-1} BAP.

The experiment with sycamore explants from *in vitro* seedlings was not successful. With the exception of callus formation at the base of the explants there was a general lack of response to the culture conditions and to a range of media and hormone concentrations (results not presented). The inclusion of activated charcoal in the medium decreased the level of callus formation, as observed in ash, but in the case of sycamore it did not have any effect on shoot growth or multiplication.

Difficulties in getting sycamore to proliferate and grow *in vitro* have been a common observation of many researchers. Many approaches have been attempted on a range of *Acer* species but success has been rarely reported. Hanus and Rohr (1985) obtained *in vitro* plants from stem explants of *Acer pseudoplatanus* L. and *A. negundo* using MS basal salts with Bourgin and Nitsch (1967) organic components and activated charcoal. Attempts to repeat their work during this project did not meet with success.

The success achieved with juvenile ash tissue is consistent with the few reports in the literature. Generally, success with *Fraxinus* species has been low. Preece *et al.* (1987) obtained plantlets from sterilised buds of *F. americana* L. (white ash) seedlings. Chalupa (1990) reported shoot proliferation of ash explant from seedling on MS and DKW media supplemented with BAP and IBA. Regeneration of shoots from embryo hypocotyls has been reported by Tabrett and Hammatt (1992). Hammatt and Ridout (1992) recommended DKW medium supplemented with 22.2 µM BA (benzyl adenine) as an appropriate medium for the micropropagation of common ash. Such reports are not inconsistent with the results reported here.

TABLE 5: EFFECT OF DIFFERENT CONCENTRATIONS OF BAP ON SHOOT MULTIPLICATION, SHOOT HEIGHT AND CALLUS FORMATION AT THE BASE OF ASH NODAL EXPLANTS.

BAP (mg L^{-1})	*Mean shoot multiplication*	*Mean shoot height mm*	*Mean callus formation*
1.0	1.33b[1]	14b	0.78b
2.5	1.62a	16a	1.56a
5.0	1.77a	10c	1.45a

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$).

TABLE 6: EFFECT OF ACTIVATED CHARCOAL ON SHOOT MULTIPLICATION, SHOOT HEIGHT AND CALLUS FORMATION AT THE BASE OF ASH NODAL EXPLANTS.

Medium	*Mean shoot multiplication*	*Mean shoot height (mm)*	*Mean callus formation*
With AC	1.00b[1]	0.9b	0.09b
Without AC	2.19a	1.7a	2.43a

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$).

3.2.2 Effects of CO_2 enrichment, basal media, conductivity levels and supporting matrix on photoautotrophic growth of ash explants in vitro.

The objective of this experiment was to find the best substrate and medium with an appropriate electrical conductivity for photoautotrophic culture of ash nodal explants. The experiment consisted of a factorial design using three different media (WPM, DKW and Enshi) at four different conductivities (full strength and diluted to give EC readings of 2.5, 2.0 and 1.5 dS m^{-1}) and four different substrates {agar, hortifoam, vermiculite and vermiculite plus gelrite (1.5 g L^{-1})}. Five replicates per treatment were used each containing five explants. Nodal explants from a single clone of *in vitro* grown seedlings were placed on shoot multiplication medium (DKW with 5 ppm BAP, 30 g L^{-1} sucrose and 7 g L^{-1} agar) in 60 ml glass jars for one week prior to transferring to the appropriate treatment in Magenta vessels with vented GA-7 lids. The cultures were placed in the photoautotrophic growth chamber with a PPFD of 90 µmol $s^{-1}m^{-2}$, 3000 ppm CO_2, a 16 hr-photoperiod at 25 ˚C. The survival rate, number of rooted explants, the number of roots, root lengths, root fresh weights, shoot elongation and shoot fresh weights were measured after six weeks.

3.2.2.1 Survival rate

Type of medium, substrate, interaction of medium and substrate, medium and EC, medium and substrate and EC all had significant effects on the survival rate of plants. In general, the survival rate was significantly higher on Enshi medium than on either WPM or DKW media (Table 7). Survival rate was highest on agar and vermiculite, the latter two substrates being significantly poorer (Table 8).

The lowest survival rate was on WPM with a conductivity of 2.5 dS m^{-1} (Table 11). Maximum survival rate was observed in Enshi medium with no significant differences between the different conductivities and substrates. The minimum survival rate of explants was observed in WPM and DKW with agar or vermiculite plus gelrite. In general, the treatments with foam and vermiculite dried out faster than those with agar, or vermiculite plus gelrite and therefore the survival rates decreased on these substrates. The deleterious effect of high conductivity in full strength WPM and DKW was most severe in the hortifoam substrate followed by vermiculite (Table 12). The naturally high conductivity was probably exacerbated by evaporation from the medium, leading to still higher concentrations of ions. In addition, it is well known that agar (and presumably gelrite) bind a large proportion of the ions in the medium in a manner that prevents them from having an effect on the plant tissue. This would mediate the effect of high ionic concentrations when such gelling agents are used.

3.2.2.2 Rooting of ash nodal explants

The type of medium and the interactions between medium and substrate and conductivity of substrate had significant effects on the percentage of rooted explants. The percentage of rooted explants was maximised in WPM and there were significant differences in this regard between WPM and Enshi, or DKW (Table 7). The maximum rooting of plants occurred on Enshi

TABLE 7: EFFECT OF MEDIUM ON SURVIVAL, ROOTING, NUMBER OF ROOTS PER PLANT, SHOOT HEIGHT AND SHOOT AND ROOT FRESH WEIGHT OF ASH EXPLANTS.

Medium	*Survival %*	*Rooting %*	*No of roots per plant*	*Shoot height cm*	*SHFW g*	*RFW g*
Enshi	98a[1]	42.3b	1.37b	2.24b	0.21b	0.074b
WPM	82b	56.4a	1.82a	2.97a	0.23b	0.10a
DKW	90b	46.2b	1.65a	2.88	0.34a	0.11a

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$), SHFW: shoot fresh weight, RFW: root fresh weight.

medium with hortifoam. DKW with vermiculite or vermiculite plus gelrite and WPM with agar, hortifoam and vermiculite plus gelrite did not have significantly fewer roots (Table 10). There were significant interactions between the conductivity and the type of medium and the percentage of rooted explants with WPM at a conductivity of 3.2 dS m^{-1}, yielding 58% rooted explants and DKW at full strength (6.1 dS m^{-1}) yielding 32% rooted explants (Table 11).

The interaction of medium by substrate by conductivity reveals that the type of substrate had the greatest effect on the number of rooted plants and the effect of high conductivity was mitigated by the type of substrate. In general the highest number of rooted plants was observed on the Enshi medium with foam (92% rooted), and DKW with vermiculite plus gelrite (88% rooted), while the lowest was on DKW with foam (Table 12).

3.2.2.3 Number of roots per plant

The type of medium and the interaction of medium and substrate and medium and conductivity had significant effects on the number of roots per plant. The maximum number of roots per plant was observed on the WPM and DKW media which were significantly higher than those produced on the Enshi medium (Table 7).

Explants placed on Enshi medium produced a maximum number of roots per explant when foam was used as the substrate. The lowest root number per plant was observed in DKW with foam (Table 10). The number of roots per plant was at a maximum in the WPM with a conductivity of 2.0 dS m^{-1} and minimum in full strength DKW (Table 11).

3.2.2.4 Length of roots

The type of substrate and the interaction of medium and substrate, substrate and conductivity and medium by substrate by conductivity had significant effects on the root lengths of ash explants. The most significant root length was observed on media in which agar was used as the substrate. The shortest root lengths were produced in hortifoam (Table 8) and the combination of DKW with hortifoam (Table 10).

Roots in the Enshi medium with agar or hortifoam were significantly longer than those produced in the vermiculite or vermiculite plus gelrite treatments. With WPM, when agar was used as a substrate, the root length was significantly longer than in hortifoam, vermiculite, or vermiculite plus gelrite. In the DKW with agar treatment, full strength medium reduced the root length compared with the length at lower conductivities. DKW with vermiculite or vermiculite plus gelrite at full strength (EC = 6.1) produced the longest roots (Table 12).

3.2.2.5 Root fresh weight

The type of medium, substrate and conductivity had significant effects on the root fresh weight produced by the explants. DKW was the best medium for maximising root fresh weight. The lowest root fresh weight was observed in the Enshi medium, which was significantly lower than WPM or DKW media (Table 7). Root fresh weight was highest when agar was used as a substrate and it was significantly different from other substrates. Root fresh weight was lowest when foam was used as a substrate (Table 8). Root fresh weight was significantly higher in full strength medium (highest level of EC for each medium) than those with a lower conductivity (Table 9).

3.2.2.6 Shoot growth (height)

The type of medium, substrate and conductivity had significant effects on shoot height of ash explants. The maximum shoot height was observed in the WPM and DKW media and these were significantly higher than on Enshi medium (Table 7).

TABLE 8: EFFECT OF SUBSTRATE ON SURVIVAL, SHOOT HEIGHTS, ROOT LENGTHS AND SHOOT AND ROOT FRESH WEIGHTS OF ASH EXPLANTS.

Substrate	*Survival %*	*Shoot height cm*	*Root length cm*	*SHFW g*	*RFW g*
Agar	98.0a[1]	2.11c	5.72a	0.25b	0.15a
Hortifoam	80.5b	2.44bc	2.47c	0.23b	0.06c
Vermiculite	78.6b	3.58a	3.37b	0.36a	0.10b
Vermiculite + gelrite	92.0a	2.65b	3.74b	0.19b	0.07c

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$).
SHFW: shoot fresh weight, RFW: root fresh weight.

The greatest shoot height was observed in the treatments in which vermiculite was used as a substrate and the lowest shoot height was observed in the media in which agar was the substrate (Table 8). There was a correlation between medium strength and shoot height with maximum shoot height occurring in the full strength media and the minimum shoot height in the media with a conductivity of 1.5 dS m^{-1} (Table 9).

The interaction of medium and substrate had a significant effect on the shoot height of ash explants, as with most of the other variables examined The WPM and DKW media with vermiculite resulted in the greatest shoot heights in the trial. The minimum shoot height was observed on Enshi medium with agar (Table 10).

3.2.2.7 Shoot fresh weight

Medium, type of substrate, the conductivity of the medium and the interaction between medium and conductivity had significant effects on shoot fresh weight. DKW medium yielded the maximum shoot fresh weight and this was significantly higher than on Enshi or WPM media (Table 7). Shoot fresh weight was highest when vermiculite was used as a substrate and it was significantly better than the three other substrates used (Table 8).

Shoot fresh weight was highest in full strength media (the highest EC) and it was significantly lower with the lower levels of conductivity. Shoot fresh weight was lowest at the lowest level of conductivity (EC = 1.5 dS m^{-1}) but it was not significantly different to that achieved at the intermediate levels (EC = 2.5 or 2 dS m^{-1}) (Table 9).

3.2.2.8 Discussion of the effect of CO_2 enrichment, basal media, conductivity level and supporting matrix on photoautotrophic growth of ash explants *in vitro*

Ash explants exhibited strong and vigorous growth on a range of media under CDE conditions without sugar and hormones in the media. Due to the wide range of interactions between the various factors, generalisations and drawing firm conclusions can be difficult. However, in general, Enshi medium was good for survival and rooting of ash explants, even though root growth was not as vigorous as with other salt mixtures. Shoot growth on Enshi, while adequate was not as vigorous as with DKW medium. High levels of conductivity tended to decrease rooting and root growth but increased shoot growth. The effect of substrate was highly interactive with conductivity and type of medium. Hortifoam was excellent for growth with Enshi medium but not with DKW. Agar maximised root length but not rooting frequency or number of roots and there was also the problem of functionality of roots produced in agar (absence of root hairs and lack of oxygen in the substrate) and the fact that long roots tend to get damaged in the transplanting process. Vermiculite is a good compromise substrate but is not very user friendly under laboratory conditions and has a tendency to dry out, as does hortifoam, in longer-term cultures.

In agreement with our results, Chalupa (1987) reported that low salt media such as WPM stimulated root development in woody plants more than the high salt MS medium. Chalupa (1990) reported between 62 to 84% rooting of shoots on WPM containing sucrose and auxin. Differences were observed in the rooting of different clones. Hammatt (1996) reported callus formation and very poor root growth in ash explants. To overcome this they exposed their explants to 21 days auxin treatment followed by transfer to an auxin-free medium. Results of this experiment indicate successful rooting of ash explants without sucrose or hormones with a higher percentage rooting than has previously been reported in conventional *in vitro* culture. In addition, losses due to contamination were minimal.

TABLE 9: EFFECT OF CONDUCTIVITY ON SHOOT FRESH WEIGHT AND ROOT FRESH WEIGHT OF ASH EXPLANTS.

EC *dS m^{-1}*	*Shoot height* *cm*	*SHFW* *g*	*RFW* *g*
Full strength	3.08a[1]	0.34a	0.13a
2.5	2.69ab	0.28ab	0.10ab
2.0	2.54b	0.23b	0.07b
1.5	2.47b	0.19b	0.08b

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$).
SHFW: shoot fresh weight, RFW: root fresh weight.

3.2.3 Effects of kinetin and NAA on rooting, bud break and shoot growth of ash cuttings

The work reported to date on shoot growth and proliferation has used nodal cuttings of juvenile material derived from in vitro germinated seeds. The reason that the majority of the published literature has used material from this source is because it is possible to obtain contamination-free cultures and it appears more amenable to manipulation of its growth pattern by the use of plant growth regulators. According to Hammatt (1996) it took 200 to 600 days after initiation of mature ash material for 42% of the shoots to root and this was accompanied by 100% contamination with *Bacillus*. In order to obtain shoot material suitable for *in vitro* culture or direct establishment of ash for microcoppicing in the PAM system, several experiments studying the effect of different media, substrate and plant growth regulators were carried out.

This experiment was part of a series examining the effects of a range of hormone medium and substrate combinations in an attempt to root mature cuttings of ash taken in September. In this particular study the effect of kinetin and NAA and Solufeed medium on rooting, bud break and shoot growth of ash cuttings was examined. The Solufeed medium supported bud break and healthy shoot growth (Table 13). No rooting of the cuttings occurred, even though callusing of the basipetal end occurred in many of the explants.

3.2.4 Effect of BAP and NAA on bud break and shoot growth of ash cuttings

The objective of this experiment was to study the effect of full and half strength DKW supplemented with BAP and NAA and combinations of BAP and NAA on rooting, bud break and shoot growth. Ash cuttings with one or two buds were taken from old trees in late August 1997 and cultured in baby jars containing full or half strength DKW without sugar and solidified with agar.

The highest number of bud breaks and greatest shoot growth was observed on media with 2.0 mg L^{-1} BAP. Although this treatment was significantly different to the control, there was no significant difference between 2.0 mg L^{-1} BAP and other plant growth regulators used in this experiment (Table 14). No rooting of the cuttings occurred during the period of the experiment.

3.2.5 Effect of auxin on rooting and shoot growth of ash semi-hardwood cuttings

The objective of this experiment was to investigate the effect of IBA and NAA on rooting of ash semi-hardwood cuttings. Cuttings were taken on the 8th of July 1997 from mature trees. Solufeed at 3.0 g L^{-1} was supplemented with different concentrations of NAA (0.00, 0.25, 0.50 and 1.00 mg L^{-1}) and IBA (0.00, 0.25, 0.50 and 1.00 mg L^{-1}). Hortifoam was used as the substrate.

Treatment of cuttings with different concentrations of IBA and NAA was not effective on rooting of cuttings, but bud break and shoot growth was significantly improved. NAA was significantly more effective than IBA on bud break (Table 15).

3.2.6 Effect of hormone and type of cuttings on bud break and shoot growth of ash

Experiments were carried out with semi-hardwood cuttings taken in the months of August and September. Cuttings were subjected to a wide range of media, hormones (both singly and in combination) and substrates. Results suggested that while this material can be introduced to culture under photoautotrophic conditions and while bud break and some shoot elongation can be achieved, the lack of rooting ability ultimately compromises the sustainability of such material in culture.

A further experiment was set up comparing the response of semi-hardwood cuttings from juvenile trees and from mature trees with softwood cuttings taken from epicormic shoots removed from mature trees on Belfield campus. The type of cutting and the interaction between hormone and type of cutting had significant effects on bud break. Bud break and shoot growth of cuttings from epicormic shoots and young trees were significantly higher than on cuttings taken from old trees. There was no significant difference between cuttings taken from epicormic shoots and young trees.

TABLE 10: EFFECT OF MEDIUM AND SUBSTRATE ON SURVIVAL ROOTING, NUMBER OF ROOTS/PLANT, SHOOT HEIGHT AND ROOT LENGTH OF ASH EXPLANTS.

Medium	*Substrate*	*Survival* %	*Rooting* %	*No. of roots per plant*	*Shoot height* cm	*Root length* cm
Enshi	*agar*	99a[1]	33c	1.21b	1.69d	5.55ab
Enshi	*hortifoam*	100a	71a	1.82a	3.31b	4.32b
Enshi	*vermiculite*	96a	38bc	1.26b	2.12cd	2.12c
Enshi	*ver + gelrite*	96a	30c	1.17b	1.85cd	2.37c
WPM	*agar*	96a	58ab	1.72a	2.54c	6.15a
WPM	*hortifoam*	76b	57ab	1.76a	2.18cd	2.03c
WPM	*vermiculite*	68b	51b	1.75a	4.18a	3.19bc
WPM	*ver + gelrite*	86b	59ab	2.05a	2.96bc	3.80bc
DKW	*agar*	99a	32c	1.56	2.11cd	5.45ab
DKW	*hortifoam*	66b	28c	1.01b	1.83d	1.05d
DKW	*vermiculite*	72b	57ab	1.97a	4.44a	4.79b
DKW	*ver + gelrite*	94ab	68a	2.06a	3.14b	5.04ab

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$), ver : vermiculite.

TABLE 11: EFFECT OF MEDIUM AND CONDUCTIVITY ON SURVIVAL, ROOTING, NUMBER OF ROOTS/PLANT AND SHOOT FRESH WEIGHT OF ASH EXPLANTS.

Medium	*EC* $dS\ m^{-1}$	*Survival* %	*Rooting* %	*No. of roots per plant*	*SHFW* g
Enshi	2.4	100a[1]	33b	1.33b	0.21c
Enshi	2.5	98ab	71a	1.36b	0.21c
Enshi	2.0	97ab	38ab	1.28b	0.21c
Enshi	1.5	97ab	30b	1.49a	0.20c
WPM	3.2	88bc	58a	1.92a	0.32bc
WPM	2.5	68bc	57a	1.71a	0.24c
WPM	2.0	82.5bc	51a	2.05a	0.21c
WPM	1.5	88.5ab	59a	1.61a	0.19c
DKW	6.1	74bc	32b	1.22b	0.52a
DKW	2.5	90ab	28b	1.72a	0.23c
DKW	2.0	78.5bc	57a	1.75a	0.43ab
DKW	1.5	88bc	68b	1.91a	0.17c

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$).
SHFW= shoot fresh weight.

TABLE 12: EFFECT OF MEDIUM, SUBSTRATE, AND EC ON SURVIVAL, ROOTING AND ROOT LENGTH OF ASH EXPLANTS.

Medium	*Substrate*	*EC* $dS\ m^{-1}$	*Survival* %	*Root length* mm	*Rooting* %
Enshi	*Agar*	2.41	100a[1]	7.3a	52c
Enshi	*Agar*	2.5	100a	6.0ab	8e
Enshi	*Agar*	2.0	100a	3.3bc	8e
Enshi	*Agar*	1.5	96ab	5.6b	24d
Enshi	*Hortifoam*	2.41	100a	5.0b	52c
Enshi	*Hortifoam*	2.5	100a	4.1b	72b
Enshi	*Hortifoam*	2.0	100a	3.8bc	92a
Enshi	*Hortifoam*	1.5	100a	4.4b	68b
Enshi	*Vermiculite*	2.41	100a	3.3bc	56c
Enshi	*Vermiculite*	2.5	96ab	0.6d	12d
Enshi	*Vermiculite*	2.0	100a	2.0c	40cd
Enshi	*Vermiculite*	1.5	92ab	2.6c	44c
Enshi	*Vermiculite + G*	2.41	100a	1.0c	16e
Enshi	*Vermiculite + G*	2.41	96a	3.9b	44c
Enshi	*Vermiculite + G*	2.0	88ab	1.9cd	28d
Enshi	*Vermiculite + G*	1.5	100a	2.6c	22d
WPM	*Agar*	3.2	100a	6.3ab	80a
WPM	*Agar*	2.5	100a	7.4a	60b
WPM	*Agar*	2.0	88ab	3.7bc	36d
WPM	*Agar*	1.5	100a	7.2a	60b
WPM	*Hortifoam*	3.2	76b	1.7cd	64b
WPM	*Hortifoam*	2.5	68bc	1.8c	60b
WPM	*Hortifoam*	2.0	92ab	2.7c	62b
WPM	*Hortifoam*	1.5	72bc	1.9c	40cd
WPM	*Vermiculite*	3.2	80ab	4.6b	44c
WPM	*Vermiculite*	2.5	52c	2.0c	32d
WPM	*Vermiculite*	2.0	56bc	3.2bc	48c
WPM	*Vermiculite*	1.5	84ab	2.9c	80a
WPM	*Vermiculite + G*	3.2	96ab	4.2b	80a
WPM	*Vermiculite + G*	2.5	52c	3.8b	28d
WPM	*Vermiculite + G*	2.0	96ab	4.3b	68b
WPM	*Vermiculite + G*	1.5	100a	2.9c	60b
DKW	*Agar*	6.11	96ab	3.5bc	36d
DKW	*Agar*	2.5	100a	6.5a	32d
DKW	*Agar*	2.0	100a	5.3b	28d
DKW	*Agar*	1.5	100a	6.5a	32d
DKW	*Hortifoam*	6.11	44c	0d	0e
DKW	*Hortifoam*	2.5	98ab	2.9c	34d
DKW	*Hortifoam*	2.0	32c	0.5d	12e
DKW	*Hortifoam*	1.5	92ab	0.8d	36cd
DKW	*Vermiculite*	6.11	76b	7.3a	60bc
DKW	*Vermiculite*	2.5	64bc	4.8b	44c
DKW	*Vermiculite*	2.0	84ab	4.7b	72b
DKW	*Vermiculite*	1.5	68bc	2.4bc	52bc
DKW	*Vermiculite + G*	6.11	84ab	7.6a	44c
DKW	*Vermiculite + G*	2.5	100a	4.7b	68b
DKW	*Vermiculite + G*	2.0	100a	3.8b	72b
DKW	*Vermiculite + G*	1.5	92ab	4.2b	88a

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$), G: gelrite.

TABLE 13: EFFECTS OF KINETIN AND NAA ON BUD BREAK AND SHOOT GROWTH OF ASH HARDWOOD CUTTINGS.

Hormone	*Bud break %*	*Shoot growth (mm)*
Control	44[2]	6.6[2]
NAA (0.2 mg L^{-1})	22	5.0
NAA (0.5 mg L^{-1})	40[2]	6.6[2]
Kin (0.5 mg L^{-1})	33[1]	8.7[3]
Kin (0.5 mg L^{-1}) + NAA (0.01 mg l^{-1})	53[3]	10.3[3]
Kin (0.5 mg L^{-1}) + NAA (0.1 mg l^{-1})	40[2]	5.5[1]
Kin (1.0 mg L^{-1})	60[3]	8.0[3]
Kin (1.0 mg L^{-1}) + NAA (0.01 mg L^{-1})	50[2]	5.0[1]
Kin (1.0 mg L^{-1}) + NAA (0.1 mg L^{-1})	40[2]	3.0
Kin (2.0 mg L^{-1})	47[2]	5.5[1]
Kin (2.0 mg L^{-1}) + NAA (0.01 mg L^{-1})	67[3]	5.5[1]
Kin (3.0 mg L^{-1})	53[3]	7.8[3]
Kin (3.0 mg L^{-1}) + NAA (0.01 mg L^{-1})	58[3]	3.7
Kin (3.0 mg L^{-1}) + NAA (0.1 mg L^{-1})	73[3]	12.0[3]

[1] $p \leq 0.05$ [2] $p \leq 0.001$ [3] $p \leq 0.0001$, Kin: kinetin.

Bud break in cuttings taken from mature trees on this occasion was negligible (Table 16). Rooting was not observed in any of the treatments within the time scale of the experiment.
The interaction between source of cutting and hormone treatment is shown in Table 17.

The level of bud break was zero or extremely low in nodal explants from mature trees irrespective of the hormone treatment applied. With explants from a juvenile source, 40% of the control explants exhibited bud break. Hormone treatment did not significantly improve this rate, indeed BAP at 3.0 ppm significantly decreased the rate of bud break. While the no hormone control with epicormic shoots was not significantly different to the juvenile source of explants, it was significantly better than direct cuttings from mature trees in respect of bud break and subsequent shoot growth. Shoot growth in the epicormic control was lower than that from the juvenile control, reflecting perhaps the greater carbohydrate reserves in the larger cuttings from the juvenile trees.

3.2.7 Effect of TDZ, BAP and GA_3 on bud break and shoot growth of dormant sycamore buds

The objective of this experiment was to obtain shoot explants for conventional tissue cultures and study the possibility of direct establishment of dormant material cultured on agrifoam under CDE conditions. Hardwood cuttings of sycamore with one or two dormant buds were taken from old trees on the 17th of December, 1997 and surface sterilised. The cuttings were treated with TDZ (0, 25, 50 and 100 ppm), BAP and GA_3 (0, 125, 250, 5000 and 1000 ppm) for 0, 1, 3, 6 and 24 hours. Cuttings were cultured in baby food jars ventilated with Sun Caps, containing DKW medium, Solufeed with 6.0 g L^{-1} agar, without sugar and placed under CDE and high PPFD conditions. The effect of plant growth regulators, concentrations, duration of hormone treatments and the interaction between hormones and concentrations on bud break were significant. In general, treatment with GA_3 induced significantly more bud break than TDZ or BAP (Table 18).

Long (24 hour) exposure times to PGRs invariably led to no bud break, except in the case of gibberellic acid treatment where significantly improved rates of bud break were observed (Table 19).

3.2.8 Establishment of in vitro stool beds

Stool beds were established for both sycamore and ash. In the case of sycamore, the route was direct establishment of semi-hardwood cuttings in Agrifoam slabs (90 x 1300 x 25 mm). These were placed either in an enclosed system in containers with ventilation and placed on the growth room shelf under photoautotrophic conditions, or they were placed in a hydroponic system with recirculating nutrients, again in the growth room under photoautotrophic conditions.
In the case of ash, microcuttings were removed from semi-hardwood cuttings that had been

TABLE 14: EFFECT OF PLANT GROWTH REGULATORS AND FULL OR HALF STRENGTH DKW ON BUD BREAK AND SHOOT GROWTH OF ASH CUTTINGS.

Medium	*Plant growth regulator*	*Bud break %*	*Shoot growth mm*
DKW	*Control*	13	3.50
DKW	*NAA 0.2 mg L^{-1}*	7	1.50
DKW	*NAA 0.5 mg L^{-1}*	13	5.00
DKW	*BAP 2.0 mg L^{-1}*	33[3]	12.00[2]
DKW	*BAP 2.0 mg L^{-1}+NAA 0.02 mg L^{-1}*	13	5.00
DKW	*BAP 2.0 mg L^{-1}+NAA 0.2 mg L^{-1}*	13	3.00
1/2DKW	*Control*	7	1.00
1/2DKW	*NAA 0.2 mg L^{-1}*	20[1]	11.50[2]
1/2DKW	*NAA 0.5 mg L^{-1}*	20[2]	11.50[1]
1/2DKW	*BAP 2.0 mg L^{-1}*	27[2]	5.50
1/2DKW	*BAP 2.0 mg L^{-1}+NAA 0.02 mg L^{-1}*	13	2.00
1/2DKW	*BAP 2.0 mg L^{-1}+NAA 0.2 mg L^{-1}*	13	1.50

[1] $p \leq 0.05$ [2] $p \leq 0.001$ [3] $p \leq 0.0001$.

subject to conditions that caused bud break and elongation. These were excised, treated with an IBA or NAA rooting powder and placed in either vermiculite, hortifoam or agrifoam slabs in enclosed containers (i.e. not a recirculating nutrient system).

Sycamore cuttings rooted readily in the agrifoam slabs and extension growth of the cuttings occurred. After approximately six weeks, apical cuttings were removed, leaving at least two nodal buds on the original cutting. Bud break and growth occurred within a further four weeks, when cuttings were again removed. This procedure was repeated over a five month period, with the cuttings being re-rooted into further agrifoam slabs. The growth of sycamore in the stool-beds established in the re-circulating system was superior to that in the enclosed system. There are probably a number of factors influencing this, including the ability to maintain the conductivity of the medium in the case of the recirculating system and the improved ventilation in this system leading to a more 'normal' type of shoot growth. The softwood microcuttings could be easily rooted under photoautotrophic conditions *in vitro* and the coppice beds yielded up to four microcuttings per plant after the third cycle of cutting. It was important not to stress the plants in the microstoolbed as this led to leaf loss and slowing of shoot growth, or in the worst case, induction of dormancy.

Initial rooting of the ash microcuttings was quite poor with only 30% rooting achieved. With successive pruning (micro-coppicing) of the cultures however, this increased to 90% success in a five week period as opposed to the 12 weeks taken to root the initial cuttings. After each pruning, bud break and stem elongation took 17 to 21 days, after which each stem had produced a further two to three nodes and the material was ready for a further pruning. The cuttings took 10 to 14 days for visible root initials to appear and had a well-developed root system by five weeks after subculture. The initial multiplication rate was one microcutting per plant but by repeated pruning the growth of more lateral shoots occurred, to yield two to three cuttings per plant by the fourth and fifth pruning. The trial was terminated after five successive sets of cuttings had been taken.

3.2.9 General discussion of shoot growth and multiplication in ash and sycamore

Direct establishment of sycamore nodal or shoot tip cuttings using plant growth regulators either by inclusion in the medium or as a short exposure (quick dip) treatment displayed differing degrees of success depending on the age of the stock plant and the season in which the cuttings were collected. More than 70% of cuttings collected from one year old glasshouse grown material rooted under CDE conditions to establish new plants, whereas this figure dropped to 40% in cuttings from newly flushed buds from field grown plants. Substrate had significant effects, with both hortifoam and vermiculite proving useful with results of 83% and 77% rooting respectively.

TABLE 15: EFFECT OF IBA AND NAA ON BUD BREAK AND SHOOT GROWTH OF ASH SEMI-HARDWOOD CUTTINGS.

Auxin	*Bud break* %	*Shoot growth* mm
NAA	52.50[1]	10.81[1]
IBA	31.25	6.50

[1] $p \leq 0.0001$

TABLE 16: EFFECT OF AGE OF STOCK PLANT ON BUD BREAK AND SHOOT GROWTH OF ASH.

Type of cutting	Bud break %	*Shoot growth* mm
Mature	4	0.3
Juvenile	30[1]	4.5[1]
Epicormic	40[1]	5.0[1]

[1] $p \leq 0.0001$

TABLE 17: EFFECT OF SOURCE OF CUTTING AND HORMONE ON BUD BREAK OF ASH CUTTINGS.

Source of cutting	*Hormone*	*Bud break* %	Shoot growth mm
Mature	*Control*	0	0.0
Mature	*NAA (0.5 mg L^{-1})*	10	1.0
Mature	*kin (0.5 mg L^{-1})*	0	0.0
Mature	*kin (2.0 mg L^{-1})*	0	0.0
Mature	*BAP (3.0 mg L^{-1})*	10	0.5
Juvenile	*Control*	40	7.0
Juvenile	*NAA (0.5 mg L^{-1})*	50	10.1
Juvenile	*kin (0.5 mg L^{-1})*	20	1.5
Juvenile	*kin (2.0 mg L^{-1})*	40	3.5
Juvenile	*BAP (3.0 mg L^{-1})*	0	0.0
Epicormic	*Control*	30	3.0
Epicormic	*NAA (0.5 mg L^{-1})*	20	5.5
Epicormic	*Kin (0.5 mg L^{-1})*	60	3.5
Epicormic	*Kin (2.0 mg L^{-1})*	30	2.5
Epicormic	*BAP (3.0 mg L^{-1})*	60	8.0

TABLE 18: EFFECT OF HORMONE TREATMENT ON BUD BREAK OF DORMANT SYCAMORE BUDS.

Hormone	*Bud Break* %
TDZ	16.24
BAP	18.74
GA_3	52.49[1]

[1] $p \leq 0.0001$

TABLE 19: EFFECT OF EXPOSURE TIME, PGR TYPE AND CONCENTRATION ON BUD BREAK OF DORMANT SYCAMORE BUDS.

PGR	*Conc. (mg L^{-1})*	*Time (hr)*	*Bud break* %	*p ≤*
TDZ	0	1	60	0.0001
TDZ	0	3	47	0.001
TDZ	0	6	40	0.05
TDZ	0	24	0	n.s.
TDZ	25	1	7	n.s.
TDZ	25	3	27	0.05
TDZ	25	6	20	n.s.
TDZ	25	24	0	n.s.
TDZ	50	1	13	n.s.
TDZ	50	3	0	n.s.
TDZ	50	6	0	n.s.
TDZ	50	24	0	n.s.
TDZ	100	1	0	n.s.
TDZ	100	3	0	n.s.
TDZ	100	6	47	0.001
TDZ	100	24	0	n.s.
BAP	0	1	67	0.0001
BAP	0	3	47	0.001
BAP	0	6	40	0.05
BAP	0	24	0	n.s.
BAP	250	1	17	n.s.
BAP	250	3	20	n.s.
BAP	250	6	7	n.s.
BAP	250	24	0	n.s.
BAP	500	1	7	n.s.
BAP	500	3	20	n.s.
BAP	500	6	0	n.s.
BAP	500	24	0	n.s.
BAP	1000	1	27	0.05
BAP	1000	3	37	0.05
BAP	1000	6	13	n.s.
BAP	1000	24	0	n.s.
GA3	0	1	60	0.0001
GA3	0	3	47	0.001
GA3	0	6	27	0.05
GA3	0	24	0	n.s.
GA3	250	1	33	0.05
GA3	250	3	40	0.05
GA3	250	6	53	0.0001
GA3	250	24	33	0.05
GA3	500	1	73	0.0001
GA3	500	3	80	0.0001
GA3	500	6	80	0.0001
GA3	500	24	33	0.05
GA3	1000	1	100	0.0001
GA3	1000	3	100	0.0001
GA3	1000	6	47	0.001
GA3	1000	24	33	0.05

n.s: not significant.

All cuttings rooted without the use of plant growth regulators, although plant growth regulators proved useful for bud break and enhancing subsequent shoot growth. These results indicated that rooting softwood cuttings from sycamore may be achieved without plant growth regulators under photoautotrophic conditions and without contamination losses and can be used as the basis for setting up *in vitro* stool beds for the multiplication of selected clones.

Direct establishment of stem cuttings collected from mature sycamore trees was successful with 87% of semi-hardwood cuttings rooting when treated with rooting hormone powder and cultured on hortifoam substrate. In general, hortifoam proved better than vermiculite as a substrate when using the quick dip method but vermiculite was superior when including PGRs in the growth medium. Cold treatment of sycamore semi-hardwood cuttings did not increase the percentage of rooting unlike the reports for *Acer palmatum atropurpureum* cuttings collected in November (Marcinkowski 1988).

Semi-hardwood cuttings proved to be the most suitable type of sycamore cutting for the direct establishment and growth of non-axenic cultures under photoautotrophic conditions. Stock plants that were heavily pruned in March produced new shoots, which were used for semi-hardwood cuttings in July. The percentage of rooting increased in these cuttings, confirming the results of Land et al. (1995) with American sycamore (*Platanus occidentalis*) where hedging stock plants was shown to rejuvenate cuttings.

Shoot elongation and further shoot initiation from established cuttings was variable in the trials carried out and application of different media did not show a consistent response or pattern. Even within treatments some buds developed vigorous extensions and growth rates while others remained dormant. This phenomenon has been previously observed in photoautotrophic woody plant culture (Long 2000). Sub-culturing only the most actively growing material leads over a number of cycles to a more homogeneous response pattern. Sycamore is particularly sensitive to stress during culture and this may be partly the cause of induced dormancy in the buds. It was noted that the leaves in new shoots produced under photoautotrophic conditions were red in colour and were slow to green, suggesting perhaps light, temperature, or even salt stress. All of these factors would be worth investigating further. The commonly used inorganic nutrients used in plant tissue culture have been optimised for mixotrophic cultures, but not photoautotrophic cultures. This point has been studied for photoautotrophic cell cultures (Horn and Widholm 1984) but not for micropropagation purposes. Kozai *et al*. (1991) have pointed out that the requirements of the two types of culture should be quite different. There is also some evidence to suggest that sycamore requires a low light intensity for shoot growth and perhaps the level of PPFD the cultures were subject to under the stated conditions had a negative effect on shoot growth.

In this study, cuttings collected from late June to October from mature trees all sprouted to different degrees, depending on the medium and substrate used. It is the first report that bud break and shoot growth can be achieved during active growth of ash trees growing under natural conditions. Silveira and Cottignes (1993) reported that stem cuttings of ash from 4 to 7 year old trees growing under natural conditions only sprouted when taken from dormant material. Apical buds taken during periods of shoot apical dormancy in September, January and March were able to sprout on a variety of media containing sucrose under mixotrophic conditions (Lloyd and McCown 1980), but no growth was obtained from apical buds removed in May or June, which is the period of cell proliferation and intense branch growth (Silveira and Cottignes 1993). In these experiments, bud break and sprouting could occur under CDE and high PPFD conditions during all the months in which cuttings were taken (June, July, August, September, November, December, January and March).

The type of medium had a significant effect on bud break, although at certain times of the year (March) bud break could be achieved by placing the cuttings in water. Generally, Solufeed proved to be particularly efficacious. In terms of substrate, in some trials hortifoam was clearly superior but in others agar or vermiculite were just as effective.

Microcuttings obtained from bud growth from a range of hardwood and semi-hardwood cuttings could be rooted and used to establish *in vitro* stool beds. At this point only 30% rooting of such cuttings had been achieved. This is considered very good as ash is considered very difficult to propagate from stem cuttings. In successive harvests from the stool beds however, the rooting percentage increased up to 90% and new cuttings could be harvested at 3 to 4 week intervals.

TABLE 20: EFFECT OF IBA AND NAA ON ROOTING OF ASH SOFTWOOD CUTTINGS.

Treatment	*Rooting*
	%
Control	0b[1]
IBA	28ab
NAA	21ab

[1]Means followed by the same letter are not significantly different, ($p \leq 0.05$).

3.3 Rooting and weaning

The final stage of any micropropagation programme is the rooting and weaning of the shoots back into *in vivo* conditions. Much of the work on establishment of cultures under photoautotrophic conditions carried out during the course of the project involved the rooting of various explants on different substrates, so a substantial body of knowledge was built up in this regard and has been reported in previous sections. In general terms both soft and semi-hardwood cuttings from sycamore could be rooted with ease. Ash semi-hardwood cuttings could not be rooted but bud break and stem elongation could be achieved, providing a source of softwood cuttings. Trials were carried out examining the effects of different hormone treatments on the rooting of softwood cuttings, which are the ultimate product of any micropropagation system.

3.3.1 Effect of IBA and NAA on rooting of ash softwood cuttings

Softwood cuttings which were regenerated from stem cuttings collected from old trees under CDE conditions in hortifoam were treated with 0.5 mg L^{-1} IBA or NAA and cultured in Magenta boxes with ventilated lids. Enshi medium was used with vermiculite as the substrate. There was no significant difference between IBA and NAA on percentage rooting, which in this case was low. Untreated cuttings failed to root (Table 20).

3.3.2 Weaning of micropropagated material of ash and sycamore

There were no large-scale replicated trials on weaning of micropropagated plants carried out during the course of this study. As plants became available from various trials and experiments, small samples were potted on and placed in the glasshouse. All material of both ash and sycamore weaned and grew with 100% survival rate under normal glasshouse conditions. These findings are in full agreement with all other studies on weaning of micropropagated material from photo-autotrophic sources. Due to a fully operative photosynthetic pathway, well-developed cuticle, functional stomata and functional roots, plants derived from cultures under photoautotrophic conditions transferred to *in vivo* conditions much more readily than those from mixotrophic conditions did.

4. CONCLUSIONS

The overall aim of this project was to investigate the potential of a photoautotrophic system for rapid clonal multiplication of ash and sycamore. While conventional mixotrophic micro-propagation of sycamore has been reported in the literature, micropropagation of ash, except from seedling tissue, has not been reported.

In the course of this project, it was demonstrated that material from mature trees can be selected in the field and introduced into culture in a manner that proved possible to use nodal cuttings to establish micro-coppicing beds directly, using simple nutrient media. Shoots were rooted into a range of substrates other than agar, the conventional substrate for micropropagation. From a practical point of view, foam substrates proved easy to use, were autoclavable, could be used a number of times and yielded good results. With ash, direct rooting of cuttings was not possible under the stated conditions and indeed this confirms reports in the literature. However, a system of inducing bud break and elongation of nodal buds was developed and it proved possible to root these shoots with a 30% success rate initially. Once the material had been growing under photoautotrophic conditions for a longer period, the success rate improved to 90%.

Proliferating nodal cultures of either ash or sycamore were not achieved. However, a multiplication system under autotrophic conditions was developed using nodal increases. This pathway is preferred in many herbaceous crops due to the fact that it functions through the growth of single nodal buds, reducing the probability of somaclonal variants, or mutants occurring. Under mixotrophic conditions, growth and nodal increase in woody plants can be slow, leading to the perception that proliferating nodal cultures, where axillary meristems within the nodal bud are encouraged to grow and develop through the use of plant growth regulators, is a more efficient pathway for producing microcuttings. Under the autotrophic conditions used in this study, sequential nodal cuttings could be removed from micro-coppices at three week intervals, at a rate faster than can be achieved using conventional cultures. In addition, if contamination and weaning losses are taken into account it is probable that the multiplication rates achieved under autotrophic conditions are equal to those possible under mixotrophic conditions.

It is obvious that the techniques developed and explored during the course of this project have immediate applications in the industrial propagation of ash and sycamore.

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APPENDIX I

	Media mg L^{-1}			
	WPM	***DKW***	***MS***	***Enshi***
Macro Elements				
$CaCl_2$	72.50	112.50	332.02	
$Ca(NO_3)$	386.80	1367.00		944.00
KH_2PO_4	170.00	265.00	170.00	
K_2SO_4	990.00	1559.00		
$MgSO_4\ 7H_2O$	180.54	361.49	180.54	492.00
NH_4NO_3	400.00	1416.00	1650.00	
KNO_3			1900.00	808.00
$NH_4H_2PO_4$				152.00
Micro Elements				
$CuSO_4\ 5H_2O$	0.25	0.25	0.025	0.12
$FeNa_2$ EDTA	36.70	44.63	36.70	19.5
H_3BO_3	6.20	4.80	6.20	2.82
$MnSO_4\ H_2O$	22.30	33.80	16.90	2.02
$Na_2MoO_4.H_2O$	0.25	0.39	0.25	0.21
$ZnSO_4.7H_2O$	8.60	17.00	8.60	0.22
$CoCl_2\ 6H_2O$			0.025	
KI			0.83	
Vitamins				
Glycine	2.00	2.00	2.00	
myo-Inositol	100.00	100.00	100.00	
Nicotinic Acid	0.50	1.00	0.50	
Pyridoxine HCl	0.50		0.50	
Thiamine HCl	1.00	2.00	0.10	

Plant tissue culture media:
WPM (McCown Woody Plant Medium, Lloyd and McCown 1981);
DKW/Juglans medium (Driver and Kuniyuki 1984);
MS medium (Murashige and Skoog 1962);
Enshi medium (cited in Kozai et al. 1988, formula supplied by Kozai).

APPENDIX II

Abbreviations

BAP	**Benzyl aminio purine**
CDE	**Carbon dioxide enrichment**
DKW	**DKW medium Driver and Kuniyuki**
EC	**electrical conductivity**
GA_3	**Gibberellic acid**
IAA	**Indole acetic acid**
IBA	**Indole butyric acid**
K	**Kinetin**
MS	**Murashige and Skoog medium**
NAA	**Napthalene acetic acid**
PAM	**Photoautotrophic micropropagation**
PGR	**Plant growth regulator**
PPFD	**Photosynthetic photon flux density**
TDZ	**Thidiazuron**
WPM	**Woody plant medium**