Optimization of Transformation in Embryogenic Cultures of Oak (*Quercus robur* L.) and Sitka Spruce (*Picea sitchensis* (Bong) Carr.).

A thesis submitted to Dublin City University in fulfilment of the requirements for the degree of Doctor of Philosophy by

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Declaration

I hereby declare that this material which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Date: 20/1/2000

This thesis is dedicated to my parents

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Abstract

Methods were investigated for the transformation of embryo cultures of Oak and Sitka Spruce with *Agrobacterium tumefaciens* and for their subsequent maturation to embryo derived seedlings.

This involved the optimization of a range of parameters which are known to have an effect on the production of transformed cells, the determination of selective conditions for the removal of *Agrobacterium tumefaciens* and for the selection of antibiotic resistant transformed cells, and investigations on the interactions of a wide range of parameters known to affect maturation of embryos to seedlings.

The isolation of cultures derived from single cell and small cell aggregates (< 14 cells) was also examined as a means to permit all cells to be accessible to *Agrobacterium* and reduce the proportion of chimeric cell colonies containing transformed and non-transformed cells.

Embryogenic cell lines were inoculated with a supervirulent strain of *Agrobacterium tumefaciens* LBA4404::pBI121. The latter contained a gene for a visible selectable marker, β-glucuronidase and a marker gene for antibiotic resistance, *npt II* gene. Transient gene expression was determined histochemically by recording the number of distinct areas of β-glucuronidase (*GUS*) activity. Experiments to optimize the transformation parameters (bacterial dilution, incubation time, co-cultivation time and acetosyringone concentration) were carried out. Maximum expression of the *GUS* gene was achieved with a bacterial suspension with an OD600 of 0.8-1.1 (c.a. 10^9 cells cm^{-3}) diluted with an equal volume of MS or Sitka Spruce embryogenesis initiation medium for Oak and Sitka Spruce respectively, inoculation of cells with bacteria for 180 min and 60 min for Oak and Sitka Spruce respectively with a 72 h co-cultivation period and exposure

of *Agrobacterium* and plant cells to 25 μM acetosyringone. Cefotaxime is the most commonly used antibiotic for the elimination of residual *Agrobacterium* following transformation. The growth of Oak and Sitka Spruce embryogenic suspension cultures in liquid medium containing the antibiotic cefotaxime was investigated. *Agrobacterium* was eliminated from cultures following co-cultivation with 500 $mgdm^{-3}$ cefotaxime antibiotic. This concentration had no effect on the plant cell growth. Growth of Oak and Sitka Spruce embryogenic suspension cultures in liquid medium containing kanamycin and paromomycin antibiotics was examined as a method of selecting cultures expressing the *npt II* gene following incubation with the *Agrobacterium* strain. Paromomycin at 30 $mgdm^{-3}$ and at 3 $mgdm^{-3}$ was strongly inhibitory to the growth of embryogenic suspension cultures of Oak and Sitka Spruce respectively and these levels were used for the selection of transformed cells following infection with *Agrobacterium*.

Regeneration of Oak embryogenic callus with a root and shoot was achieved on P24 maturation medium 1 in the light. This contained 1% Activated charcoal without plant growth regulators. Cultures were previously cultured on P24 medium plus 0.9 μM BAP. Sitka Spruce embryonal suspensor masses were easily regenerated into seedlings using culture of embryos on embryo germination medium and embryo development medium from previously published reports..

Cultures derived from single cells from both Oak and Sitka Spruce embryogenic suspension cultures were produced by exposing cultures to a pectinase ($0.07gdm^{-3}$) digestion. Sitka Spruce single cells required a Percoll density separation (19%) step to separate the single cells derived from the embryogenic head from those derived from the suspensor cells.

Oak single cell cultures could be more easily achieved by reducing the BAP concentration in MS medium by 100-fold to 0.01 $mgdm^{-3}$. Single cells were regenerated

back to micro-calli by increasing the BAP concentration to 1.0 $mgdm^{-3}$. Oak single cells were transiently transformed in a 50% *Agrobacterial* dilution for 60 min with a 72 h co-cultivation period with both *Agrobacterium* and plant cells exposed to 25 μM acetosyringone. *Agrobacterium* was eliminated with 500 $mgdm^{-3}$ cefotaxime antibiotic. Cultures derived from single cells provide a source of cells for transformation which can be regenerated to micro-calli and subsequently to embryos without the problems associated with protoplast cultures.

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Abbreviations

ABA	abscisic acid
AC	activated charcoal
BAP	benzylaminopurine
bp	base pairs
β	beta
cm	centimetres
cm^3	centimetres cubed
DNA	deoxyribonucleic acid
dm	decimetres
dm^3	decimetres cubed
ESM	embryonal suspensor masses
EDTA	ethylenediamine tetraacetic acid
e.g.	for example
et al.	and others
fr.wt	fresh weight
g	gram
g	*gravity*
GA_3	giberellic acid
h	hour(s)
IBA	indoyl butric acid
i.e.	that is
kb	kilobase pairs
kPa	kilo pascal
log	logarithm (common)
M	molar

µM	micromolar
mg	miligram
µg	microgram
ml	mililitre
µl	microlitre
min	minute
MS	Murashige and Skoog medium
NB	Nutrient Broth
nm	nanometre
no.	number
npt	neo phosphotransferase
O.D.	optical density
PEG	polyethylene glycol
Per. Comm.	Personal communication
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
s	second(s)
SCV	settled cell volume
SDS	sodium dodcyl sulphate
TDZ	thidiazuron
Tris	tris (hydroxymethyl) aminoethane
U.K	United Kingdom
U.S.A	United States of America
U.V.	ultraviolet
WPM	Woody Plant Medium
X-glu	5-bromo-4-chloro-3-indoyl β-D-glucuronic acid

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Chapter 1

General Introduction

1.1 The Economic Importance of Wood

It is difficult to overstate the ecological and economic importance of wood. The total amount of wood in the worlds forests is estimated at about 1.5 Gt (Gammie, 1981), making wood by far the most abundant component of the terrestrial biomass. The ecological importance of wood is vast as the carbon stored in wood and humus is important in the planetary carbon cycle which has a significant influence on global climate. Wood is a leading industrial raw material and an important component of the global economy.

1.1.1 Economics of Hardwoods and Softwoods

1.1.1.1 *Quercus* Species

The genus *Quercus* includes about 450 species with a large number of varieties and hybrids. They have a wide distribution throughout the temperate regions of the northern hemisphere in Europe, North America and Asia. In Europe Oaks represent about 9% of the growing stock in forests and cover 25–30% of the forested area in some European countries (France, Greece, Romania, U.K. and Belgium). *Quercus* species provide crops of major importance to forest and horticultural industries (Gingas and Lineberger, 1989). Oaks provide fine hardwood valued not only because of its great strength and durability but also for its beauty. The wood of Oak is hard, heavy and strong and has a pronounced growth ring figure (Chalupa, 1995). Oak wood is used in the furniture and construction industries. *Quercus acutissima* is one of the most valuable tree species used for fuel and tool handles (Kim *et al.*, 1994). *Q. acutissima* also has great importance in Japan for maintaining the resources of bed logs for Shiitake mushrooms (Shoyama *et al.*, 1992). *Quercus suber* the

cork Oak, is a forest species present in many countries of the Mediterranean basin where it is exploited for cork production (Manzanera *et al.*, 1993). Many Oak species are also grown as ornamental trees often producing attractive autumn fall colours. *Q. robur* is valued both for timber and as an ornamental (Vieitez *et al.*, 1985).

1.1.1.2 Conifer Species

Sitka Spruce (*Picea sitchensis* (Bong) Carr.) has been described as an economically important conifer native to North America. It has however become the most extensively planted exotic species in Great Britain after its introduction by Douglas in 1831. It is primarily a coastal species but in certain areas of its distribution where optimal environmental conditions exist it extends inland along river valleys. It is now considered the most important forestry species in the United Kingdom (Drake *et al.*, 1997). It has a highly persistent single leading shoot and the stem form is consistently good producing a high versatile softwood timber and very good paper pulp (John *et al.*, 1995).

A growing population is reducing the land area available for wood production in the U.S.A. and world-wide, while increasing the demand for wood products. The global demand for wood increased rapidly between 1952 and 1978 and projections indicate continued growth in demand to the year 2030. However, during the same time the productive forest area world-wide is expected to decrease dramatically (USDA. Forest Service, 1982). The growing demand on a diminishing resource therefore results in the need for greater productivity of remaining forest lands (Whetten *et al.*, 1991).

1.1.1.3 Forestry in Ireland

The Irish National forestry inventory is worth at least £130 million. There are currently 606,000 hectares of forestry in the state with an estimated 21% broadleaf cover.

Afforestation in Ireland is currently running at 30,000 acres per year compared with a target of 60,000 set down by the National Forestry State (Mac Connell, 1999). There has been an over reliance on Sitka Spruce in the Irish Forestry Industry to make the industry commercially viable and to support some 16,000 jobs (O' Sullivan, 1998). The estimated harvest valuation of 30 year old Sitka Spruce is in the region of £5,000 per acre, whereas an acre of 5 year old Sitka Spruce would reach a value of £1,500, making it one of the most significant tree species in the country.

1.2 Genes for Trees

The potential for tree crop improvement are the introduction of virus resistance, insect resistance, toxin resistance, freezing resistance and genes for wood quality and form.

1.2.1 Virus Resistance

There are three strategies for introducing virus resistance into plants 1). using antisence viral RNA 2). cross protection using genes encoding the viral coat protein 3). expression of satellite RNA. The expression of viral antisence has produced limited success in restricting virus infection and/or disease development (Beachy, 1991). Widespread success has been achieved in producing virus resistant plants through the expression of genes that encode viral coat proteins. Beachy, (1991) developed resistance against different classes of virus in the crop Alfalfa (*Medicago sativa*). This approach has been successful against a number of different viruses e.g. tobacco mosaic virus (TMS) (Bevan *et al.*, 1985) and plum pox virus (PPV) (Machado *et al.*, 1992). Satellite RNA has the ability to modify the virulence phenotype of their companion viruses. Transgenic

plants expressing the satellite RNA of the tobacco ring spot virus (TobRV) show less severe symptoms when infected by the corresponding virus (Gerlach *et al.*, 1987).

1.2.2 Insect Resistance

Plants with resistance to certain insects have been produced by employing genes which code for protease inhibitors and for insecticidal crystalline proteins (ICPS). Although these mechanisms are different they both work by disturbing the digestion of the food.

The Bt gene or *Bacillus thuringiensis* delta endotoxin is the most commonly used insect resistance gene. Preparations of *Bacillus thuringiensis* are already being used in the U.S.A. as bioinsecticide sprays in forestry (Doughlas, 1995). This insecticidal activity of *B. thuringiensis* resides in the spore of the bacterium. It occurs as a crystalline inclusion body containing one or more insecticidal inclusion bodies (ICPS) which are also known as delta endotoxins (Dandekar *et al.*, 1994). When the ICP is ingested by the target insect it is first solubilised in the alkaline pH of the insects midgut and then acted upon by proteases which release the insecticidal crystal fragment (ICPF) and this kills the insect. ICPFS are encoded by *cry*IA (c) and *cry*IA (b) genes and are known to be lethal to key target walnut insect pests (Leple *et al.*, 1993). Transgenic Apple for the Bt gene has been obtained (James *et al.*, 1993). There is also a potential use for insect resistance through the Bt gene in transgenic pine, spruce and Douglas fir to afford protection against the large pine weevil (Douglas, 1995). Resistance to phytopathogenic bacteria can be induced by introducing genes encoding insect humoral immunity proteins such as cecropins in plants (Steikema, 1992). Bacterial and fungal genes including cecropins and chitinases have been cloned and are being use to develop disease tolerant deciduous trees (du Pleiss, 1993). Shin *et al.*, 1994 produced transgenic European larch (*Larix decidua* Mill.) plants expressing a *Bacillus*

Thuringiensis Berliner (B.t) toxin gene or the glyphosate tolerance (*aro*A) gene using *Agrobacterium rhizogenes*-mediated transformation.

1.2.3 Toxin Resistance

Toxic substances, for example herbicides and heavy metals, interfere with normal cellular function. Resistance to a toxin can be achieved by three types of mechanisms described by Comai and Stalker, 1986: 1). the altered target mechanism 2). overexpression 3). detoxification. Creation of glyphosate resistance transgenic plants is an example of the altered target strategy. Glyphosate is a herbicide marketed by the Monsanto Company as Roundup, which is toxic as it interferes with biosynthesis of aromatic amino acids. Mutant *Salmonella typhimurium* were obtained which displayed resistance to glyphosate the gene, which had been mutated, was *aro*A. The *aro*A gene was used to make a gene which could be expressed in plants.

1.2.4 Freezing Resistance

Genes encoding proteins based on the antifreeze protein found in Arctic flounder hold the potential for decreasing freezing damage both in crops and cold-stored produce. These have been introduced into Tobacco and Tomato (Hightower *et al.*, 1991). Von Schaevan *et al.*, 1990 showed increased cold tolerance in Tobacco plants expressing *E coli* pyrophosphatase.

1.2.5 Genes for Wood Properties

Lignin is an aromatic polymer and one of the chief substances found in wood. Lignin was once regarded as a waste product in paper manufacturing but now is used as fuel in paper pulp mills. It is also used in making plastics, fertilizers, artificial vanilla,

cosmetics and rubber. In the paper process industry lignin has to be removed from cellulose in a toxic and energy consuming process involving sodium sulphite or sodium hydroxide treatment (Boerjan *et al.*, 1995). Thus it would be of obvious benefit to process trees with less or a modified lignin which would prove easier to separate from cellulose. Boerjan *et al.*, 1995 found that the inhibition of CAD (cinnamylalcohol dehydrogenase) activity in Poplar increases lignin extractability.

1.2.6 Genes for Tree Form

The plant hormone group the cytokinins has been shown to regulate the process of cell division (Skoog & Miller, 1957) to release axillary buds from apical dominance (Sachs and Thiman, 1964) and to delay senescence (Richmond and Lang, 1957). An elegant method of regulating these processes could be achieved by the introduction into the plant genome of a cytokinin gene controlled by a tissue specific, developmentally or environmentally regulated promoter. A number of genes whose expression results in cytokinin production have been isolated and sequenced from various strains of the plant pathogen *Agrobacterium tumefaciens* (Barry *et al.*, 1984; Beaty *et al.*, 1986). Each of these genes codes for the enzyme isopentyl transferase (*IPT*) (Akiyoshi *et al.*, 1984). Smigoghi *et al.*, 1993 reported enhanced resistance of *Nicotiana* to tobacco hornworm and green peach aphid with the introduction of the *IPT* gene. Hammerschlag and Smigoghi, 1994 transformed Peach with *IPT* gene and reported that there was a 1.5-6.6 fold increase in the number of axillary shoots in the transformed plants. It was thought that the introduction of this cytokinin biosynthesis gene may be a useful approach to obtaining Peach trees with a compact growth habit.

When plants are transformed with a series of *rol* genes of *Agrobacterium rhizogenes*, characteristic effects on apical dominance, adventitious rooting and the rate and habit of root development have been induced.

1.3 Genetic Engineering of Woody Species

In the last ten to fifteen years a variety of methods have been discovered for introducing foreign genetic material into plant cells. There are two main plant transformation techniques: 1). Vectored transformation by *Agrobacterium* or viral vectors 2). Direct transformation using naked DNA delivery.

1.3.1 *Agrobacterium*-mediated Transformation

Agrobacterium is a small micro-organism that is capable of infecting a broad assortment of dicotyledonous plants after they have been wounded. Woody angiosperms and gymnosperms have been successfully transformed or genetically engineered using a variety of *Agrobacterium* vectors (Parsons, 1986; Fillati *et al.*, 1987; De Verno and Cheliak, 1988; Confalonieri *et al.*, 1994; Bekkaoui *et al.*, 1990; Roest & Evers, 1991; Howe *et al.*, 1994; Ellis *et al.*, 1989; Shin *et al.*, 1994; Loopstra *et al.*, 1990).

The main limitation of *Agrobacterium* has been the apparent inability to transform monocots. Attempts to extend *Agrobacterium* host range to include these species involved testing new bacterial strains, varying host genotype, manipulating explant physiology, modifying co–cultivation conditions and use of superior selectable markers (Godwin *et al.*, 1992). Deliberate wounding of explants, the most commonly used *Agro*–infection methodology to date is that published by Horsch *et al.*, 1985. Modification of this approach have been adapted to several other species. Many laboratories all over the world

are using *Agrobacterium tumefaciens* as a vector for routine production of transgenic crop plants (Songstad *et al.*,1995).

1.3.1.1 Methods of *Agrobacterium* Infection

In terms of plant transformation using *Agrobacterium* vectors we see that there are two species of *Agrobacterium, Agrobacterium tumefaciens* and *Agrobacterium rhizogenes.* These bacteria induce crown gall and hairy root disease respectively at wound sites on dicotyledonous plants. A few monocotyledonous plants for example members of the families *Lilaceae* and *Amaryllidaceae* have been found to be weakly susceptible to crown gall induction. Once initiated, this tumorous growth can continue in the absence of the bacterium and the tumour tissue and grow axenically in tissue culture media which lacks exogenous supplies of auxins and cytokinins which under normal conditions are required to promote growth of plant tissue *in vitro* (Draper *et al.*, 1988). This tumour tissue produces novel amino acids and sugar derivatives which are collectively known as opines.

Tumour induction along with opine synthesis is associated with the presence of a mega plasmid, the Ti plasmid within the bacteria. In the case of *A. rhizogenes* the plasmid is referred to as the Ri plasmid. The utility of *Agrobacterium* as a gene transfer system was first recognized when it was demonstrated that the crown galls were actually produced as a result of the transfer and integration of genes from the bacterium into the genome of the plant cell (Gasser *et al.*, 1989).

These Ti mega plasmids are found in all virulent strains of *A. tumefaciens* and are found to be 200 to 250 kb in size. These plasmids are stably maintained in *Agrobacterium* at temperatures below 30°C. Genetic analysis of this Ti plasmid has shown that there are two regions, the T-DNA and the Vir region which are associated with tumour formation (Draper *et al.*, 1988).

During tumour formation a defined sequence of the Ti plasmid, the T-DNA, is transferred to the plant cell and integrated into the plants own nuclear genome. This transferred T-DNA was found to be stable in the plants genome. This phenomenon was discovered by the hybridization of the Ti plasmid specific probe to tumour DNA. Therefore by virtue of the fact that the T-DNA in the plant cell is co linear with the T-DNA in the Ti plasmid of the *Agrobacterium* no major rearrangements of the sequence takes place during re-establishment of the tumour. It is possible to have one or more copies of the T-DNA in the plant DNA and although multiple copies of T-DNA can occur in tandem repeats they may also be separated and linked to different regions of the plant DNA. It appears that the site of integration of T-DNA into the plant DNA is random. An interesting point to note is that *Agrobacteria* that are cured of their Ti plasmid were no longer able to induce tumours (Caplan *et al.*, 1983). This supports the statement that the plasmid is involved in tumour formation.

The Ri plasmid found to be present in the *Agrobacterium rhizogenes* strain is not used as frequently as the Ti plasmid in plant transformation experiments. Under the control of a virulent region two separate T-DNA regions of the Ri plasmid are transferred to the plant genome (Hoffman *et al.*, 1984; De Paolis *et al.*, 1985). These regions are termed the Tl (T left T-DNA) and the Tr (T right T-DNA). The Tr T-DNA contains genes for opine production and strains have been found to be characterized by their particular opine genes (De Paolis *et al.*, 1985). The Tr T-DNA also contains two genes which code for auxin synthesis which appear to be highly homologous to the auxin genes of *A. tumefaciens*. The Tl T-DNA does not have homology with the T-DNA of *A. tumefaciens* (Hoffman *et al.*, 1984). It has been found that strains of *Agrobacterium* possessing both the Tl and the Tr T-DNA are more virulent on a wider range of plant species, than are strains possessing only one T-DNA (Vilaine and Casse-Delbart, 1987).

The Ti plasmid carries a number of genes which are involved in both plant and bacterial function. The most important of these genes is the Vir gene. The Vir gene products cause T-DNA to be transferred to the plant cell nucleus where it is integrated into the genome (Lewin, 1990).

The T-DNA regions in both *A. tumefaciens* and *A. rhizogenes* are flanked by 25bp direct repeats and the end points of integrated T-DNA in the plant genome are found to be close to these sequences (Draper *et al.*, 1988). The T-DNA itself is found to contain 8-13 genes including a set for the production of phytohormones which are responsible for the formation of the characteristic tumours when transferred to infected plants. The majority of studies that have been carried out on the mechanism by which T-DNA is transferred from *Agrobacterium* to the plant cell have been done with *A. tumefaciens.*

Experimental results suggest the following model for transformation. *Agrobacterium* comes in contact with the compounds released from the wounded plant tissue transcription of the Vir region of the Ti plasmid occurs. One specific chemical highly active in this respect has been identified as acetosyringone (Stachel *et al.*, 1986). The Vir region of the Ti plasmid codes for six genes that are responsible for transfer of T-DNA to an infected plant, these are Vir A, Vir B, Vir G, Vir C, VirD and Vir E (Lewin, 1990). Vir A and Vir G are expressed in vegetatively growing bacteria although Vir G is only transcribed at a low level. When *Agrobacterium* is exposed to a wounded plant cell exudates or simply pure acetosyringone the Vir A gene product is thought to recognize and interact with it and transmit the extracellular signal intracellularly resulting in the activation of the Vir G gene product. The altered Vir G protein then activates the rest of the virulence genes as well as elevating transcription from the Vir G locus. This Vir G induction is followed by the appearance of single stranded nicks within the 25bp border sequences which flank the T-DNA (Stachel *et al.*, 1986 ; Albright *et al.,* 1987) and the appearance of

a single stranded linear molecule which corresponds to the T-DNA. The products of the Vir D operon are thought to be similar for this specific endonuclease activity (Yanofsky *et al.*, 1986). By a mechanism then which remains unknown but is thought to be analogous to bacterial conjugation the T-DNA is transferred to the plant cell and stably inserted into the nuclear DNA (Stachel and Zambryskii, 1986).

These properties of *Agrobacterium* as a natural genetic engineer make it highly suitable for use as a transformation vector for transfer of novel heterologous genes to plants. It is now known that *Agrobacterium* can be used very efficiently and effectively as a transformation vector. It was shown that the natural ability of *Agrobacterium* to transfer defined sequences of DNA into the plant genome could be exploited in the development of a variety of plant transformation vectors. These vectors capitalize on several inherent characteristics of the *Agrobacterium*-mediated transformation process. The main features of *Agrobacterium* that makes it amenable to exploitation in the construction of plant transformation vectors are:

1). The onc genes are not required for the transfer of T-DNA to the plant cell and its integration within the nuclear genome 2). The Vir region of the Ti and Ri plasmid functions in trans 3). DNA inserted between the 25 base pair border repeats of the T-DNA whether the borders are natural or synthetic is transferred to the plant cell 4). No apparent rearrangements of the DNA located between the T-DNA borders takes place during the transfer to the plant genome 5). The foreign DNA integrated into the plant genome can be stabily inherited in a Mendelian manner (Walden, 1988).

The onc genes are not needed in the process of T-DNA transfer therefore these genes can be replaced not only allowing the insertion of foreign DNA but also removing these onc functions. To date a limit on the insert size has not been reported (Draper *et al.*, 1988).

Using these Ti plasmid vectors any foreign DNA which has been inserted or cloned can be transferred into the genome of a dicotyledonous plant cell. The foreign DNA which is to be transferred must be flanked by the T-DNA border sequence and stably maintained in an *Agrobacterium* harbouring a full complement of Vir genes either in cis or in trans. The removal of the onc functions means that transformed tissue is no longer recognizable as neoplastic outgrowths which can be selected by their ability to grow on a medium lacking phytohormones.

Non oncogenic vectors that are commonly in use can be divided into two types i.e. cis and trans depending on whether the T-DNA region flanked by 25 base pair direct repeat sequences are carried on the same replicon as the Vir genes or on a separate plasmid. The former, the cis acting Vir genes are commonly referred to as co-integrative vectors whilst the latter with the trans acting Vir genes are known as binary vectors. The co-integrative vectors are vectors which are based on a wild type Ti or Ri plasmid from which portions of the T-DNA have been removed or replaced by a novel sequence of DNA. Often with the Ti plasmid the region which has been removed encodes the onc function and this allows for the regeneration of normal non-tumourous plants using conventional procedures of tissue culture as already mentioned. These vectors are often called disarmed vectors since they are based on the Ti or Ri plasmid, they are stable within the *Agrobacterium* and because they retain the Vir region and therefore have all the apparatus needed for the transfer of sequences which are located between the border repeats of the co-integrative vector (Walden, 1988). The binary or trans vectors are based on plasmids which are capable of replicating both in *E.coli* and strains of *Agrobacterium* and that contain the T-DNA border sequence flanking multiple cloning sites as well as markers that allow direct selection of the transformed plant cell. These binary vectors allow manipulation in *E. coli* followed by transfer to *Agrobacterium* by conjugation in the presence of a helper plasmid (Hoekema *et*

al.,1983). Binary vectors are capable of functioning in both *A. rhizogenes* and *A. tumefaciens* (Simpson *et al.*, 1986). This vector replicates as an independent plasmid in *Agrobacterium* and the transfer of the foreign DNA into the plant cell is mediated by the Vir region of the resident Ti or Ri plasmid acting in trans.

The choice of vector system for a particular transformation procedure depends on many factors not least of which is the aim of the experiment and the particular species involved. Many vectors have been designed with a particular plant in mind and hence it is important to select a vector carefully for the task required of it. It is important to understand that no one vector system can be used in every circumstance particularly with regard to host range as many were originally tested using only model transformation systems for example those often used are Tobacco and Petunia and almost without exception *Solanaceous* species. There are a number of specific vectors available based on the Ti or Ri plasmid examples of some more commonly used vectors are SeV vector system and the Bin19 vector.

1.3.2 Viral Vectors

Plant DNA viruses have received attention as vectors for introduction of foreign genes into plants. The interest is derived from the fact that viruses as pathogenic agents normally enter the plant cell, express the information contained in their genomes and can replicate to achieve a high copy number (Walden, 1988). In 1984 propagation and expression of bacterial selectable marker genes transferred to plants by cauliflower mosaic virus (CaMV) were first demonstrated (Brisson *et al.*, 1984). More recently the transforming capacities of the Ti-plasmid-derived and viral vectors have been combined to create a technique called *agro*-infection. The introduction of maize streak virus (MSV) into maize and of the separate A and B components from tomato golden mosaic virus

(TGMV) into Petunia have been reported for this technique (Harrison, 1985). RNA viruses that replicate only through RNA intermediates may also be used as vectors for plant genetic engineering.

Viral vectors provide some advantages for the introduction of foreign genes into plants. These include the ease of infection, a different host range to *Agrobacterium* and a high number/expression rate of inserted genes under the control of appropriate promoters. A number of disadvantages can also be pointed out. The maximum size of passenger DNA insertable without affecting viral infectivity may be limited. Viral infections usually result in specific disease symptoms or a lethal to the plant. There is also a possibility of high error frequency during viral RNA synthesis which may lead to incorrect expression of the inserted gene.

1.3.3 Free DNA Delivery Systems

1.3.3.1 Microprojectile Bombardment

One of the most significant developments in the area of cell transformation with naked DNA has been high velocity microprojectile technology. In this system DNA is carried through the cell wall and into the cytoplasm on the surface of small metal particles that have been accelerated one to several hundred meters per second (Gasser *et al.*, 1989). These metal particles range in size from 0.5–5 μm. These particles are capable of penetrating through several cell layers allowing the transformation of cells within tissue explants.

The first report of microprojectile bombardment to deliver DNA to living cells was by Klein *et al.*, 1987. DNA was delivered by discharging a 0.22 caliber cartridge to accelerate tungsten microprojectiles carrying DNA through an evacuated chamber and into

the cells. The first transgenic plants and progeny via this method were with tobacco (Klein *et al.*, 1988; Tomes *et al.*, 1990). The main advantage of this system for DNA delivery is that unlike *Agrobacterium* it is suitable for a variety of monocots such as Barley (Wan and Lemeux, 1994) and Rice (Christou *et al.*, 1991). Microprojectile bombardment has been used to enhance *Agrobacterium* infection in Sunflower (*Helianthus annus* L.) (Bidney *et al.*, 1992). Transient gene expression in pollen of Norway Spruce (*Picea abies*) was achieved by particle acceleration (Martinussen *et al.*, 1994). Charest *et al.*, 1996 achieved stable genetic transformation of *Picea mariana* (Black Spruce) via particle bombardment of cotyledonary somatic embryos and suspensions from embryonal masses. Stable transformation of White Spruce and regeneration of transgenic plants through microprojectile bombardment of somatic embryos was achieved (Bomminieni *et al.*, 1993; Ellis *et al.*, 1993). Bomminieni *et al.*, 1994 also reported expression of a *gus* gene in somatic embryo callus of Black Spruce (*Picea mariana*) after microprojectile bombardment.

1.3.3.2 Microinjection

Microinjection is an old technology dating to the 1880's. Microinjection of plant cells however is far from routine and it is a technique more readily associated with infection of animal cells. Plant cells are much more difficult to infect than animal cells for two reasons. Firstly, plant cells have a cell wall composed of relatively thick layers of pectin, hemi-cellulose and cellulose that are difficult for a glass microneedle to penetrate. Secondly, the vacuole contains many hydrolases and toxic compounds. If the vacuolar contents are emptied into the cytoplasm the cell will most likely die. There are many reports however demonstrating that protoplasts can survive microinjection (Griesbach, 1985; Lawrence and Davies, 1985; Mowikawa and Yamada, 1985; Reich *et al.*, 1986a).

Crossway *et al.*, 1986 microinjected *Agrobacterium* plasmid DNA into tobacco plants using a technique known as the hold pipette technique. Duchesne and Charest, 1992 studied the effect of promoter sequence on transient expression of the β–glucuronidase gene in embryonic calli of *Larix x eurolepis* and *Picea mariana* following microinjection.

1.3.3.3 Electroporation

Electroporation is the use of high field strength electrical pulses to make cell membranes permeable in a reversible manner to facilitate transfer of DNA directly into cells (Fromm *et al.*, 1986). There are two important factors affecting the process 1). the strength of the electric field which in turn depends on 2). the diameter of the protoplast and the pulse decay time. The first reports describing DNA delivery to intact electroporated tissue were by Abdul-Baki *et al.*, 1990 and Mathews *et al.*, 1990 using Tobacco pollen. Zaghmout and Trolinder, 1993 reported a method of electroporating *Agrobacterium* plasmid DNA into Wheat callus cells.

1.3.3.4 Electrophoresis

Ahokas, 1989 introduced DNA into intact embryos by designing an electrophoretic chamber in which the cathode was connected to a pipette containing DNA. After electrophoresis, radioactive labelled DNA could be observed throughout the apical meristem and transient gene expression was observed. Electrophoretic-mediated DNA delivery is influenced by various physical factors associated with plant tissue. These include the frictional co–efficient (f) which is a physical property of the cell wall and plasma membrane. Within an electrical field DNA migrates through the cellulose fibers of the cell wall (Dekeyser *et al.*, 1990; Lu and Ferl, 1992) and passes through the plasma

membrane. *Calanthe orchid.* L., zygotic embryos were stably transformed following electrophoresis (Griesbach and Hammond, 1993).

1.3.3.5 SAAT–Sonication Assisted *Agrobacterium* Transformation

SAAT is a new process in which target tissue which is immersed in *Agrobacterium* suspension is subjected to ultrasound (Santarem *et al.*, 1998). The enhanced transformation rates using SAAT probably result from the microwounding, where the energy released by cavitation causes small wounds both on the surface and deep within the target tissue. SAAT has been shown to enhance *Agrobacterium* mediated transformation of Soybean (Trick and Finer, 1997).

1.3.3.6 Silicon Carbide Whisker-Transformation.

DNA delivery into plant cells using silicon carbide whiskers was investigated by Kaepplar *et al.*, 1990. The method is very simple and involves vortexing a mixture of plasmid DNA encoding screenable and selectable markers, silicon carbide fibers and plant cells to be transformed. Silicon carbide fibers probably mediate DNA delivery because of their shape, size, strength and chemical composition. These fibers are single crystals with an average diameter of 0.6 μm and length ranging from 10–80 μm. It is likely that improvements in DNA technology via silicon carbide fibers and plant tissue culture methods will increase the number of species that are amenable to this transformation procedure. Silicon carbide fiber DNA delivery requires very little preparation prior to DNA delivery. The procedure is rapid and inexpensive and may be used on most cell types provided that some prior investigation to optimize DNA delivery parameters is conducted. The advantage of this process is its simplicity and flexibility. The primary disadvantage however relates to its similarity to asbestos and the presumed health risk potential

(Songstad *et al.*, 1995). Frame *et al.*, 1994 reported the production of fertile transgenic Maize plants by silicon carbide mediated transformation. Wang *et al.*, 1995 found that materials with characteristics similar to silicon carbide whiskers such as silicon nitride whiskers can also deliver DNA into plant cells.

1.3.3.7 Protoplast-mediated Transformation

Protoplasts are in principal ideal cells for DNA delivery and selection of transgenic events. Removal of the cell wall eliminates a major barrier to DNA delivery. The most commonly used procedure for direct DNA delivery into protoplasts involves treatment with polyethlene glycol (PEG) to alter plasma membrane properties by causing reversible permeabolization that enables exogenous macromolecules to enter the cytoplasm. The first reports of direct DNA delivery and stable transformation involved transfer and expression of *Agrobacterium tumefaciens* T-DNA genes into tobacco protoplasts via PEG treatment (Draper *et al.*, 1982). The first example of transgenic plants was reported by Pazkowski *et al.*, 1984. Electroporation involves subjecting protoplasts to electrical pulses of high field strength to cause reversible permeabolization of the plasma membrane enabling macromolecule delivery. Stable transformation of Tobacco plants regenerated from electroporated protoplasts involved combinations of PEG and electroporation treatments (Negrutiu *et al.*, 1987). The primary advantages of electroporation over PEG or other chemical mediated treatments are reproducibility or high frequency DNA delivery and simplicity of the technique (Jones *et al.*, 1987).

1.4 Micropropagation of Woody Species

Micropropagation describes the numerous systems through which asexual or clonal propagation occurs from explants of the original plants. The main objective to micropropagation is the production of large numbers of genetically identical individuals.

1.4.1 Organogenesis

Organogenesis refers to the developmental process where organ primordia, such as buds are initiated on an explant in response to the application of exogenous hormones. Organogenesis can occur either directly from explants or indirectly by differentiation of shoot and/or root meristems in callus culture (Sharp *et al.*, 1983). Usually angiosperms respond more favourably to *in vitro* organogenesis than conifers. Nevertheless most species of trees, gymnosperms, or angiosperms temperate or tropical can be propagated via organogenesis (Cheliak *et al.*, 1990).

The system of axillary shoot multiplication was used for the micropropagation of various Oak species: *Q. robur* and *Q. petraea* (Chalupa, 1979, 1985b, 1988, 1990, 1993; Vieitez *et al.*, 1985; Meier-Dinkel, 1987; Meier-Dinkel *et al.*, 1993; Evers *et al.*, 1993) *Q. suber* (Bellarosa, 1981, 1989; Manzanera and Pardos, 1990) *Q. serrata* (Ide and Yamamoto, 1987). Chalupa 1981 discovered that a low salt nutrient medium stimulated rapid growth and proliferation of axillary shoots of Oak. Drake *et al.*, 1997 produced successful adventitious shoot regeneration from Sitka Spruce cotyledons by a cytokinin pulse method.

1.4.2 Somatic Embryogenesis

Somatic embryogenesis has been regarded as the *in vitro* system of choice with the potential for mass propagation of superior and genetically engineered forest tree genotypes in both coniferous and hardwood species (Gupta *et al.*, 1991).

The earliest work on somatic embryogenesis was conducted on carrot cultures and a substantial number of investigators have utilised this plant. Carrot has therefore become the proverbial model system and much of our understanding of somatic embryogenesis has come from work with this plant.

The ability to raise somatic embryos in cell culture creates a number of opportunities which are not available when plants are regenerated via organogenesis. One distinct advantage is that somatic embryos are bipolar structures bearing both root and shoot apices. In organogenesis root and shoot development are often mutually exclusive and a sequence of media changes is necessary to generate an entire plant. Embryo cultures can produce large numbers of embryos per culture flask, many more than the multiple shoots generated adventitiously via embryogenesis (Ammirato, 1983).

Zygotic embryos are those formed by the fertilized egg and zygote whereas somatic embyros are defined as those formed by sporophytic cells either *in vitro* or *in vivo*. Ammirato, 1983 described somatic embryogenesis as a process analagous to zygotic embryogenesis but in which a single cell in a small group of vegetative cells are the precursors of the embryos. It is supposed that given the precision of the zygotic embryo pattern formation programme, the first stages of both zygotic and somatic embryos could be similar or at least very close. A comparison however, between zygotic and somatic embryogenesis can only be established from the globular stage onwards from which a parallel evolution occurs. Despite the similarities between the two types of embryogenesis

two main differences exist, namely the lack of differentiation of endosperm and suspensor tissue in the case of the somatic system (Dodeman *et al.*, 1997).

Sharp *et al.*, 1980 described two routes for somatic embryogenesis. The first is direct embryogenesis where embryos initiate directly from tissue in the absence of callus propagation and this occurs through pre-embryonic determined cells (PEDC) where the cells are already committed to embryogenic development and need only to be released. The second is indirect embryogenesis where some cell propagation is required. This occurs in differentiated non-embryogenic cells or induced embryogenic determined cells.

Whether the differential ability of somatic cells to become embryogenic reflects genetic differences or whether it is due to the presence of a specific responsive cell type is not clear (De Jong *et al.*, 1993).

1.4.2.1 Factors Effecting Somatic Embryogenesis

A number of variables have been identified which effect somatic embryogenesis. The choice of explant from which to initiate somatic embryos is of vital importance. For a number of species only certain parts of the plant body may respond in culture. This is indeed the case for a number of moncotyledonous and graminacious species. Somatic embryogenesis has been induced in several Oak species from a variety of explants such as male catkins (*Q. bicolor* (Gingas ,1991)), leaf segments from adult trees (*Q. ilex* (Feraud-Keller and Espagnac, 1989), anthers (*Q. robur* (Chalupa, 1985b)), immature zygotic embryos (*Q. rubra* (Gingas and Lineberger, 1989), *Q. cerris* (Ostrolucka and Pretova, 1991), zygotic embryo segments (*Q. lebani* (Srivastava and Steinhauer, 1982).

Proper explant selection has been reported to be critical in order to achieve successful induction of somatic embryogenesis in conifers (Tautorus *et al.*, 1991). In general in *Pinus* species precotyledonary zygotic embryos (*Picea glauca, Picea mariana*

(Tremblay, 1990; Tautorus *et al.*, 1990a) are best for induction of embryogenic tissue whereas in *Picea* species cotyledonary zygotic embryos are best (Becwar *et al.*, 1988). This also indicates the importance of the time of explant collection and suggests that there is a developmental period which exists in which zygotic embryos are highly committed to forming embryogenic tissue.

The type of culture medium used has a large affect on the initiation of somatic embryos. Somatic embryos have been grown in a wide variety of media types. It is known that a source of amino acids plays a role and L-glutamine appears to be the most favoured. Tautorus *et al.*, 1991 reported that modifications to medium components and culture conditions can significantly affect induction of embryogenic tissue and play a major role in enhancing initiation from more mature plants (von Arnold, 1987; Attree *et al.*, 1990; Simola and Santanen, 1990). The most important factors are concentration of basal medium, nitrogen level, mineral elements, agar and pH.

The presence of growth regulators in the culture medium has a large affect on the development of embryonic tissue. The presence of auxins alone or in a combination with cytokinin appear essential for the onset of growth and induction of embryogenesis (Fujimura and Komamine, 1980a). *Quercus* is in the list of species which does not require auxin for the induction of somatic embryos. Formation of Oak somatic embryos can occur in the absence of exogenous hormones, however in most cases the development of somatic embryos is stimulated by manipulation of hormonal balance in the nutrient medium. However in all cases of somatic embryos initiated from immature and mature zygotic embryos the presence of cytokinin is required and in most cases was present in the form of benzylaminopurine (BAP). The presence of 2,4-D proved to be the best plant growth regulator for somatic embryo induction experiments in *Q. robur* (Mazanera, 1993) compared with combinations of BA and NAA. Bonneau *et al.*, 1994 noted that for the

European Spindle tree, somatic embryos were never observed when the explants were incubated in medium in the absence of a cytokinin. Sucrose is reported to be the most effective reduced carbon for somatic embryogenesis. Ammirato, 1983 reported that sucrose is the most effective carbon source and osmoticum for somatic embryogenesis in most angiosperm species. Sucrose is also important for influencing further development of embryos past the globular stage by affecting developmental synchrony or abnormal morphology (Michler *et al.*, 1991).

Environmental conditions such as light and dark have proved important and somatic embryogenesis has occurred under a variety of light/dark regimes.

The culture regime may also play a significant role in somatic embryogenesis and in particular its maintenance. The embryogenic capacity of cultures has been seen to decrease and disappear through progressive subculturing (Syono, 1965) and this loss of potential has been traced, at least in certain cases, to a change in chromosomal complement where aneuploids gradually replace diploids (Smith and Street, 1974). The maintenance of chromosomal and genetic integrity is essential if the goal of somatic embryogenesis is clonal reproduction. There are a number of studies demonstrating that frequent subculturing can effectively minimize the effect of chromosomal changes in cell cultures (Bayliss, 1977; Evans and Gamborg, 1982). Therefore careful attention to the subculture regime may help maintain genetic and chromosomal viability.

1.4.2.2 Somatic Embryogenesis in *Quercus* Species

Reports on somatic embryogenesis in *Quercus* provide evidence that Oak tissue is highly embryogenic. Oak somatic embryos originate in embryogenic tissue through a series of developmental stages. The initiation of somatic embryos in *Quercus* is greatly dependent on the type of explant used. Immature zygotic embryos are often used as

explants and have been shown to be highly embryogenic. Embryogenic cultures of *Quercus robur* and *Quercus petraea* were originally initiated from immature zygotic embryos (Chalupa, 1985b, 1990, 1993) as were embryogenic cultures of *Quercus rubra* (Gingas and Lineberger, 1989), *Quercus serrata* (Sasamoto and Hasori, 1992). Chalupa reported that repetitive embryogenesis was frequent for *Q. robur* and led to the formation of many small embryoids. Embryogenic tissues maintained in media containing cytokinin retained their embryogenic potential. Media most frequently used for embryo initiation in *Quercus* were Murashige and Skoog (Murashige and Skoog, 1962) and Woody Plant Medium (Lloyd and McCown, 1980).

1.4.2.3 Somatic Embryogenesis in Conifer Species

Three different processes have been suggested that could account for the origin of conifer somatic embryos (Hakman *et al.*, 1987). Somatic embryos may arise from single cells or small cell aggregates by an initial asymmetric division that delimits the embryonal apex and suspensor region. Somatic embryos may develop from small meristematic cells within the suspensor or somatic embryos could arise by a mechanism similar to cleavage polyembryogenesis with the initial separation occurring in the embryogenic region. Tautorus *et al*, 1991 reported that the best results for somatic embryogenesis were obtained using immature and mature zygotic embryos as the source of the tissue and this is true for *Picea sitchensis*. Dunstan *et al.*, 1988 devised a numbering system which he used to describe the various stages of somatic embryo development. Stage 1 embryos were described as a repressed pro-embryo with translucent suspensor and semi translucent, densely staining embryo with an irregular outline. Stage 2 embryos are prominent and smooth in outline, opaque cream to pale yellow in colour and subtended by a suspensor. Stage 3 embryos have primordial cotyledons clustered around a central meristem and are

cream to pale green in colour. Finally stage 4 embryos have distinct, partly elongated cotyledons clustered around a central meristem and are green in colour. Later stages of development included elongation of both cotyledons and hypocotyls and the development of a radicle occurring are referred to as germinating embryos.

Somatic embryogenesis offers a great potential for the large-scale production of forest trees. The formation of totipotent cells from embryogenic tissue (Durzan and Gupta, 1987; Michler *et al.*, 1991) may also prove useful in the production of transgenic forest trees. Millions of somatic embryos will be needed for reforestation and bioreactors can be used to produce the necessary plant cells on a large scale. Different groups have already been growing for example Radiata pine (Smith, 1991) and Interior Spruce and Black Spruce (Tautorus, 1992) in bioreactors. The production of mature cotyledonary embryos under bioreactor conditions is however a problem (Gupta *et al.*, 1993). Moorhouse *et al.*, 1996 reported on the Braun Biostat BF2 bioreactor system which was used to control somatic embryogenesis in liquid cell culture. The bioreactor was inoculated with a suspension culture of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) known to be embryogenic and capable of maturing into plantlets on solidified medium. The perfusion capability of the bioreactor was employed to replace the initial proliferation medium with maturation medium order to induce the development of somatic embryos in submerged cell culture. The cell line was found to mirror only the initial elongation previously observed in shake flask culture. The limitations of using somatic embryogenesis technology for the main propagation of elite tree species are the inability to initiate embryogenic callus from non-embryogenic tissue, low frequency of embryo formation, low germination rates, inability to control aberrant morphology and the difficulty in acclimatising germinated plantlets to *ex-vitro* environments. Once these limitations are overcome regeneration by somatic embryogenesis can be used for an array of biotechnological applications.

1.5 Somatic Embryo Maturation and Plantlet Regeneration.

The relatively poor conversion rate of somatic embryos to plantlets remains a problem for the commercial utilization of this technology. Maturation in most cases involves a series of complex media changes.

Somatic embryos of *Picea abies* (Gupta and Durzan, 1986), *Picea mariana* (Hakman and Fowke, 1987a), *Picea taeda* (Gupta and Durzan, 1987a) were matured on media with reduced or eliminated phytohormones. In all cases however plantlet formation was sporadic and continued growth infrequent. Abscisic acid (ABA) was found to play an important role in conifer embryogenesis (Gupta *et al.*, 1993). ABA inhibits cleavage polyembryony thus allowing embryo singulation, further development and maturation. The addition of ABA improved embryo maturation in a number of conifers (Durzan and Gupta, 1987; Dunstan *et al.*, 1988, 1991; von Arnold and Hakman, 1986; Attree *et al.*, 1990b) when applied prior to transfer to phytohormone free medium for final germination. Researchers have used activated charcoal to adsorb the growth regulators before transfer to developmental and maturation medium with ABA.

In terms of embryonal suspensor masses (ESM) most authors agree that to mature the embryos beyond stage 1 requires a change to the medium used. Krogstrup *et al.*, 1988 reported complete development of *Picea sitchensis* embryos from zygotic embryos to ESM to emblings growing in the soil. He used BMG-1 and modified BMG-2 supplemented with a range of combinations of kinetin, BA, ABA and 2,4-D. He also found that ABA played a major role in synchronising development. Birt, 1991 made an exhaustive study of the effect of plant growth regulators on the maturation of ESM that had been initiated. She also found that ABA had a profound effect on maturation and that it was enhanced by the

absence of light. The duration of ABA treatment may be more important than its concentration. Treatment for too short a period of 2 weeks resulted in the development of a large number of isolated roots without associated shoot meristems. For the full maturation of somatic embryos to stage 4, treatment of ABA for at least 6 weeks appears to be necessary.

Attempts have been made to mature tissues in liquid culture. This however has resulted in poor maturation and may be due to the physical disruption of the nutrient and plant growth regulator gradients within the tissues.

The serious problem of Oak regeneration via somatic embryogenesis is either an absence or a low frequency of somatic embryo conversion into plantlets. The development of somatic embryos is often blocked after the formation of cotyledons. Various treatments are therefore required for further development into plantlets.

Cytokinin concentration has an effect on embryo maturation. Chalupa reported a poor frequency of somatic embryo conversion into plantlets for *Q. robur*. He found that high cytokinin levels in the form of BAP hindered plantlet formation. Low cytokinin concentration and culture in the light led to greening and further embryoid formation. Sasaki *et al.*, 1988 and Shoyama *et al.*, 1992 reported conversion of *Q. acutissima* somatic embryos into plantlets was stimulated on Woody Plant Medium (WPM) containing a low concentration of BA. Root formation occurred on transfer to half strength WPM supplemented with IBA. Tsvetkov, 1998 succeeded in converting somatic embryos from immature embryos of *Q. robur* L. by first subjecting them to MS plus 1 $mgdm^{-3}$ BAP alone or in combination with 1 $mgdm^{-3}$ GA_3. Mature somatic embryos successfully germinated after being transferred to WPM medium plus 0.2 $mgdm^{-3}$ BAP. These embryos showed further development and some converted into plantlets.

Desiccation and dehydration treatments inside sterile sealed dishes for 2-3 weeks increased the frequency of *Q. robur* embryo conversion. Chilling treatments of *Q. petraea* embryos at 2-3 °C for 3-4 min improved their germination frequency (Chalupa, 1995). The conversion of *Q. suber* embryos to plants was also promoted by cold treatment (Bueno *et al.*, 1992; Manzanera *et al.*, 1993).

It is clear that a considerable amount of research is still required to improve the process of maturation and conversion of somatic embryos in woody species.

1.6 Selection of Transgenic Plants

One of the most important advances in the area of plant genetic transformation has been the development of genetic markers. Vital to the whole plant transformation process is the ability to show that the introduced foreign DNA has been successfully integrated into the plant genome and is expressing itself.

A gene fusion system can be defined as DNA constructions (performed *in-vitro* or *in-vivo*) that result in the coding sequences from one gene (reporter) being transcribed and/or translated under the direction of the controlling sequence of another gene (controller) (Jefferson, 1987). Many genes in plants exist in multi–gene families where products are very similar but can be regulated differentially during development of individual members of multi–gene families are often apparently inactive. The use of these gene fusions to individual members of such families with the introduction of these fusions into the genome allows the study of individual genes which are distinct from the background of the other members of the gene family.

Analysis of mutationally altered genes in plants accessible to transformation techniques is greatly facilitated by the use of sensitive and versatile reporter genes. By

using a reporter gene that encodes an enzyme activity not found in the organism being studied, the sensitivity with which heterologous gene activity can be measured is limited only by the properties of the reporter enzyme, the quality of the available assays for the enzyme and the availability of a suitable promoter. The gene fusion system chosen should be easy to quantify and highly sensitive thus allowing analysis of genes whose products are of moderate and low abundance. The reporter enzyme should be detectable with sensitive histochemical assays to localize gene activity in particular cell types (Jefferson *et al.*, 1987). The reaction catalyzed by the reporter enzyme should be sufficiently specific to minimize interference with normal cellular metabolism and general enough to allow the use of a variety of novel substances to maximize the potential for fusion genetics and *in-vivo* analysis.

1.6.1 Reporter genes

Seven reporter genes have been frequently used in studies of expression in higher plants. The *E. coli* β–galactosidase (Helmer *et al.*, 1984) enzyme have been of little use mainly due to the difficulties in performing assays. This is due to the fact that plants themselves contain high endogenous β–galactosidase activity. Use of the *Agrobacterium tumefaciens* Ti-plasmid encoded genes nopaline synthase (Depicker *et al.*, 1982; Bevan *et al.*, 1983) and octopine synthase (DeGreve *et al.*, 1982) promised to overcome problems associated with endogenous activity because the opines produced by these gene are not found in normal plant cells. Parsons *et al.*, 1986 in their *Agrobacterium* mediated transformation of Poplar utilized the opine reporter genes. These reporter genes are not widely used because the assays are cumbersome and can be difficult to quantify and they cannot be used to demonstrate enzyme localization. The two most useful reporter genes up to 1987 were the bacterial genes chloramphenicol acetyl transferase (CAT) and the

neomycin phosphotransferase (NPT II) which encode enzymes with specificities not normally found in plant tissue (Fraley *et al.*, 1983; Herrara–Estrella *et al.*, 1983a, b). However both CAT and NPT II are relatively difficult, tedious and expensive to assay. The firefly luciferase gene has also been used as a marker in transgenic plants (Ow *et al.*, 1986). This enzyme is however labile and difficult to assay accurately. Martinussen *et al.*, 1994 used the luciferase gene as a marker for the transformation by particle acceleration of Norway spruce cultures.

By far the most widely used reporter gene since its discovery in 1987 by Jefferson *et al* has been the *E. coli* β-glucuronidase gene. It has been the choice of reporter gene for a number of woody plant transformations (Roest *et al.*, 1991; Bomminieni *et al.*, 1993; Li *et al.*, 1994; Yibrah *et al.*, 1994; Frame *et al.*, 1994; Charest *et al.*, 1996; Drake *et al.*, 1997; Santarem *et al.*, 1998). β-glucuronidase fits all the criteria for a successful reporter gene system for use for transformation of plants. β-glucuronidase (GUS, EC 3.2.1.31) is encoded by the *μid*A locus and is a hydrolase that catalyses the cleavage of a wide variety of glucuronides many of which are available commercially as spectrophotometric, flurometric and histochemical substrates. β-glucuronidase is easily, sensitively and cheaply assayed *in-vitro* and can be assayed histochemically to localize activity in cells and tissues.

1.6.2 Selectable Marker Genes

The selectable functions on most general transformation vectors are procaryotic antibiotic resistance enzymes which have been engineered to be expressed constitutively in plants (Draper *et al.*, 1988). Genes affording resistance to antibiotics are the most commonly used marker genes. Examples are genes such as kanamycin G418 (Herrera-Estrella *et al.*, 1983; Bevan, 1984), hygromycin (Van den elzen *et al.*, 1985) and bleomycin (Hille *et al.*, 1986). To be of use the selectable agent concerned must be able to exert a

stringent selection pressure on the plant tissue concerned. The selectable marker most frequently used is *npt* II (neomycin phosphotransferase) which confers resistance to kanamycin. Selection on a kanamycin containing tissue culture medium gives a selective advantage to those cells that have stably incorporated the transgene construct and are therefore resistant to kanamycin. The kanamycin resistant gene continues to express in regenerated plants.

1.7 Problems Associated with Genetic Engineering of Plants

A number of problems have been associated with the incorporation of foreign DNA into plant genomes. Research indicates that desirable new phenotypes created by the introduction of foreign DNA into plants are frequently unstable following propagation, leading to the loss of newly acquired traits. Transgenic plants can only be of value if their engineered phenotype is faithfully transmitted through subsequent generations in a predictable manner. The expression of transgenes can vary considerably between different independently transformed plants (Hobbs *et al.*, 1990; Jefferson *et al.*, 1990; Blundy *et al.*, 1991). Several factors relating to integration and structure of transgene DNA such as number of transgene copies, position in the genome, and methylation, may greatly influence expression of transgenes.

The phenomenon of transgene silencing was initially described in model plants such as tobacco and *Arabidopsis* transformed by *Agrobacterium tumefaciens*. Recently however transgene silencing has been observed in a number of transgenic plants produced by particle bombardment and in economically important crop species (Walter *et al.*, 1992; Srivastava *et al.*, 1996) although it has been seldom studied in detail (Kumpatla *et al.*, 1997). The higher incidence of transgene copy numbers may cause transgene silencing to

be more frequent in plants transformed by particle bombardment than plants transformed by *Agrobacterium*.

There is evidence of interactions between different transgenes, where DNA sequences on one construct can interfere with the expression of transgenes on another (Matzke *et al.*, 1989). Other evidence carried out by molecular analysis of the promoter of transgenes which have been down regulated or switched off often reveal methylation of cytosine nucleotide residues. It is however not clear whether methylation is a cause or a consequence of gene inactivation (Jorgenson, 1993).

Another problem associated with the insertion of foreign genes into plants is the difficulty with DNA insertion into the desired position or organelle. Site specific insertion is a task which has previously proven to be difficult, in general the DNA tends to be inserted randomly into the genome. However recent studies by McBride *et al.*, 1995 on the production of an insecticidal protein in tobacco has succeeded in the specific insertion and amplification of a chimeric *Bacillus* gene in the chloroplast of the plant.

Abnormal expression of transgenes in many instances may be detected in the first generation of transgenic plants. In some cases transgenic silencing is not observed until large scale field trials (Brandle *et al.*, 1995). All of the above evidence suggests that long term studies are needed to analyze the expression and inheritance of transgenes through several generations of genetically engineered plants.

1.8 Environmental Problems and Public Concern over Genetically Modified Plants

Transformation systems are now available in a wide variety of plant species and examples of transgenes for pest, viral, fungal, and herbicide resistance and environmental stress tolerance exist. With these new developments however comes the problem of gene escapes. The fear that genes inserted will spread to wild populations or in the case of bacteria spread giving them an advantage or disadvantage in their natural environment has now become a real issue.

When considering the use of *Agrobacterium* as a tool in genetic engineering it is now imperative to address the magnitude of risk posed to the environment in releasing transgenic plants (Barrett *et al.*, 1997). Moreover newly formed combinations of persistently transmitted viruses and the opportunistic and systematically moving *Agrobacterium* vector infectious to a wide host range may eventually cause infection and damage to crop plants of natural vegetation not presently visited by the traditional vectors (insects) of the viral disease (Mogilner *et al.*, 1993). It is therefore imperative that all bacteria are eliminated prior to release of these transgenic plants.

The selectable marker most frequently used in transgenic plants is *npt* II which confers kanamycin antibiotic resistance. The kanamycin gene continues to express in the regenerated plants and would be present in any transgenic crop variety derived from them. Risk assessments of selectable marker genes have to date focused largely on the *npt* II gene in the large part because of its presence in Calgene Flavr SAVR tomatoes (Flavell *et al.*, 1992). In the health and safety arena one of the major apprehensions with the commercialisation of transgenic products has been the concern that selectable marker genes

or their products may be toxic or allergenic when consumed. Additionally when selectable markers for antibiotics which have clinical or veterinary applications are used, the concern has been raised that the marker gene could be transferred into micro-organisms and increase the number of resistant pathogenic micro-organisms in the human or animal gut (Yoder *et al.*, 1994). In the area of environmental safety the following concerns have been raised 1) a marker encoding either antibiotic or herbicide resistance may change the transgenic plant into a weedy pest 2) horizontal transmission of the marker into wild relatives may transform them into weedy pests 3) the spread of the selectable marker into other organisms may upset the balance of the ecosystem (Gressel, 1992; Dale, 1992; Nap *et al.*, 1992). There is however considerable evidence to show that the presence in crop plants of the kanamycin resistance gene does not present any risk to human health or the environment (Calgene, 1990; Bryant and Leather, 1992; Nap *et al.*, 1992).

A number of transformation systems have been developed allowing marker gene elimination. **Co-transformation** of two separate DNAs, one incorporating a gene of interest and the other the selectable marker gene may prove a simple system for the elimination of selectable marker genes provided two criteria are fulfilled. The efficiency of co-transformation needs to be high and the co-transformed DNAs must integrate at genomic locations sufficiently linked to allow effective recovery of recombination events. **Site specific recombination systems** can be used to eliminate selectable markers as first demonstrated using the *Saccharomycees cereviseae* 2 μm circle site specific recombination system. Cregg and Madden, 1989 cloned *S. cereviseae ARG4* gene between the asymmetric inverted repeat sequences (*FRT*s) which are the substrates for the site specific recombinase FLP. The *ARG4-FRT* construction was transformed into an *arg4* mutant of the yeast *Pichia pastoris* and transformants selected by Arg+ prototrophy. A plasmid expressing FLP was then introduced in a second transformation and a recombination event

was identified by selecting for the loss of Arg prototrophy. **Tissue specific expression of selectable marker genes**: In principle it should be possible to regulate transcription of the selectable marker gene by using a promoter which is preferentially expressed, either temporarally or spatially, at the site of transformation. This would allow selection of transformants without expression of the marker in mature plants. **Target gene replacement:** Ideally, a transformation system would result in the chromosomal replacement of an endogenous gene with the corresponding transgene. Homologous recombination occurs frequently in some eucaryotes and some transformation systems exploiting the mechanisms have been successfully employed in fungal and animal systems (Timberlake & Marshall, 1989; Joyner, 1991). Unfortunately gene replacement in plants is disappointingly inefficient (Halfter *et al.*, 1992).

The possibility that transgenes might be transferred to by cross pollination to sexually compatible wild plant species (Dale, 1992) also arises. If a transgene confers pest resistance, disease resistance, a tolerance to stressful growth conditions there may be a possibility of conferring a selective advantage to wild populations including weed species. There is also the possibility for transgenes which confer resistance to specific herbicides to be transmitted to wild species (Dale *et al.*, 1993). The extent of gene transfer to wild populations depends on various factors; the crop plant and the wild species must be sexually compatible they must be growing in the same area and they should flower at the same time and they should have a means of transporting pollen from one to the other.

Bacillus thuringiensis (Bt) protein has been used as a natural herbicide by organic farmers for many years. The gene which produces the Bt protein has been isolated and inserted into a number of plants enabling them to produce their own Bt toxin. Maize and cotton have been produced using this Bt gene. A number of problems however have developed. Research has found that Bt used in Maize behaves differently from natural Bt

and has a detrimental effect on insects such as lace-wings which are normally the farmers natural ally. If pests acquire immunity to Bt toxin the system will no longer work. Conventional farmers will have to switch to another system of pest control and organic farmers will have lost their only means of controlling aphids and other plant pests. Potatoes have now been engineered to commit suicide if they are infected by disease. This could reduce the need to use pesticides on potato crops. If the potatoes are attacked by fungi, the infected cells destroy themselves to avoid spreading the disease. The gene being used is the Barnase gene and scientists say that it will not be spread to wild relatives by cross pollination as the Potato plant being used is sterile under field conditions (Peerenboom, 1998).

Herbicides to which resistance is being developed are also causing concern. Crops have been developed which are resistant to three broad spectrum herbicides, glyphosate, glufosinate and bromoxynil. While companies attempt to portray these chemicals as environmentally friendly it has been shown that gyphosate is one of the top ten chemicals in terms of worker illness in agriculture in California (Genetic Concern proposal, 1999). Bromoxynil manufactured by Rhone-Paulenc is a proven mutagen and is readily absorbed through the skin. Both the Californian and U.S environmental protection agencies have increased worker protection requirements for the use of bromoxynil. Gufosinate (BASTA®) is also causing concerns due to its presence in Novartis Bt maize. Koyama *et al.*, 1994 reported that a 59 year old woman who injected a herbicide containing glufosinate suffered severe toxicity of the herbicide.

Transformation methods currently used require that transgenic plants are produced under tissue culture conditions in the laboratory. Their transgenic status is confirmed by assaying for expression of the transgenes inserted. Stable integration and the number of copies of the inserted DNA are confirmed by Southern hybridization. Following initial

analysis transgenic plants need to be moved into a containment glass house for further phenotypic and genotypic analysis using the original non-transgenic genotype as a control. In order to evaluate transgenic plants under agronomic conditions it is necessary to carry out a field assessment or field release. Because our experience of gene transfer from isolated organisms is new it is widely accepted by research scientists, plant breeders, environmental scientists, commerce and members of society generally that there is a need to carry out a risk assessment exercise before each novel type of transgenic plant is grown in small scale field trials and before they are used in transgenic crop varieties. In recognition of this need for risk assessment, release of transgenic plants are overseen by various regulatory authorities which operate in different countries.

In the United States in 1999 a total of 72 million acres of land were planted with soybean half were planted with genetically modified (GM) herbicide resistant seeds. The commercial use of GM crops in the U.S. since 1993 has taken place over comprehensive scientific reviews and approval by regulatory processes in the U.S. Department of Agriculture and Environmental protection Agency. World wide in 1999 about 28 million hectares of transgenic plants are being grown (Abelson and Hines, 1999). Reports have shown that concern over GM foods is much greater in Europe than in the US (Gaskell *et al.*, 1999). In Europe factors such as concerns over mad cow disease, dioxin contamination in animal feeds, lack of effective and transparent regulatory oversight and mistrust of government and large organizations appear to promote current furore. In order to allay these fears it is important that scientists realise that times have changed and that there is a need for them to engage in dialogue with the public rather than retreat. It is important to convey to the public that the great majority of reputable scientists working in the field consider both the processes and the products of agricultural biotechnology to be beneficial to the environment and safe for the consumer (Beachy, 1999).

1.9 Study Aims

Agrobacterium tumefaciens is capable of infecting a large number of hardwood and softwood species. Both *Quercus robur* L. and *Picea sitchensis* have been shown susceptible to a number of A*grobacterium* strains. Somatic embryogenesis has been successfully achieved for both *Quercus* and conifer species. The major obstacle for *Agrobacterium*-mediated transformation of woody species using somatic embryos as the explant is the low frequency of embryo conversion to plantlet. The aims of the current study were as follows:

- To investigate the sensitivity of Oak and Sitka Spruce embryogenic suspension cultures to a variety of antibiotics used for the elimination of *Agrobacterium* and the selection of transformants.
- To optimize a method of transient *Agrobacterium*-mediated transformation of Oak and Sitka Spruce embryogenic suspension cultures.
- Successful transformation of somatic embryo cultures is of no use if embryo conversion to plantlet cannot be achieved. Investigations into the use of a variety of media types to improve the efficiency of Oak somatic embryo conversion to plantlet.
- The study also investigated the production of cultures of single cells derived from Oak and Sitka Spruce embryogenic suspension cultures. *Agrobacterium* must contact with a plant cell surface in order for transformation of the plant cell to take place. *Agrobacterium* therefore can only transform cells at the surface of embryos, and this both limits the efficiency of transformation and ensures that the embryos which have been transformed are chimeric with a high proportion of non-transformed cells. The transformation of single cells and small cell aggregates should yield greater numbers of transformed cells and reduce the number of chimeras.

Chapter 2

Materials and Methods

2.1 Materials

Investigations into growth, maturation and transformation of Oak were carried out with the embryogenic cell line CEF, initiated from immature zygotic embryos of pedunculate Oak (*Quercus robur* L.). This embryogenic cell line was donated by Dr. David Thompson at Coillte Laboratories, Newtownmountkennedy, Co. Wicklow.

Investigations into growth, maturation and transformation of Sitka Spruce were carried out with *Picea sitchensis* (Bong.) Carr. Embryonal suspensor masses were raised from immature embryos of Sitka Spruce cones. Two-cell lines 183E and 12B were donated by Dr. Thompson. All bacterial strains were kindly donated by Dr. John Draper at the the University of Aberythswith,Wales, Great Britain. All chemicals of analytical-grade or molecular biology-grade were purchased from the Sigma-Aldrich Chemical Co. (Poole, Dorset, U.K.), BDH (Poole, Dorset, U.K.) or from Boerhringer Mannheim (Plant DNA Isolation Kit, 1667319) (Lewes, East Sussex, U.K.). Restriction endonucleases were purchased from New England Biolabs Inc. (Beverley Massachusetts, U.S.A.). All antibiotics and Murashige and Skoog basal salt mixture were purchased from Duchefa Biochemie BA (Haarlem, Netherlands). The Tomy 35SS autoclave was purchased from Mason Technologies (Dublin, Ireland) and all gyratory shaker tables were purchased from New Brunswick Scientific Company Incorporated (Edison, New Jersy, U.S.A.) and (Gallenkamp). Olympus BX40 right field microscope was purchased from the Olympus Optical Company, Japan. The CCD type colour video camera was purchased from Victor Company, Japan Ltd, Japan.

2.2 Methods.

2.2.1 Sterilization Procedures.

2.2.1.1 Heat-stable materials

All materials unless otherwise stated were sterilized by autoclaving. To conserve sterility flasks were stoppered with a cotton wool bung and wrapped in a double layer of aluminium foil. Items were autoclaved in a TOMY SS-325 autoclave at 103.4 kPa (121°C) for 20min. Larger volumes required longer sterilization times.

2.2.1.2 Heat-labile materials

All heat-labile materials such as glutamine, benzylaminopurine, rifampicin, vancomycin, pectinase, kanamycin, cefotaxime, paromomycin, abscisic acid, indol-3yl-butric acid and giberellic acid were filter sterilized through a pre-sterilized 0.2 μM pore Acrodisc™ 32 (PALLGelmann Laboratories, Dun Laoire, Co. Dublin, Ireland). Autoclaved medium was cooled to 42°C in a water bath prior to addition of filter sterilized compounds. Filter sterilization of medium did not alter the pH of the medium.

2.2.1.3 Plant material

All plant material which we received was already in culture and therefore considered sterile. However endemic bacteria were present in the cultures and antibiotics were therefore routinely used for two years to eliminate endemic contaminants (cefotaxime and vancomycin at 50 $mgcm^{-3}$).

2.2.1.4 Resterilization of heat stable material during the course of an experiment.

Scalpels and forceps were resterilized by immersing the tips of the instruments for 7s in the sterilization pot of a bead sterilizer maintained at 230°C. Materials which could not be convieniently resterilised in the bead steriliser were resterilised by passing through a roaring Bunsen flame.

2.2.1.5 Conservation of sterility

Immediately after autoclaving all materials were transferred to a laminar airflow cabinet (Gelmann Sciences). All sterile manipulations were then carried out in this cabinet. Prior to the commencement of any work in the cabinet, it was thoroughly swabbed with 70% ethanol. The bead sterilizer and bunsen burner were situated in the laminar airflow cabinet.

2.2.2 Preparation of Media

2.2.2.1 General conditions

Unless otherwise stated all media were adjusted to pH 5.7 with molar NaOH or HCL prior to autoclaving. For solidification of media, 6 gdm^{-3} of Gelrite was then added. After autoclaving solid medium approximately 20 cm^3 was dispensed into sterile 9 cm Petri dishes with a media dispenser (Accuramatic-5). For liquid medium approximately 50 cm^3 was poured into sterile 250 cm^3 Erlenmeyer flasks. Both plates and flasks were stored at 4°C until required.

2.2.2.2 Oak (*Quercus robur* L.) embryogenesis maintenance medium

Oak embryogenic callus and suspension cultures were maintained on modified Murashige and Skoog medium (MS) (Murashige, T. and Skoog, F., 1962). Table 2.1 outlines the ingredients. Vitamins were prepared in a stock solution (H-Vitamins) (Table 2.2) and 10 cm^3 was added to 1 dm^3 of MS medium. Glutamine (200 $mgdm^{-3}$) and benzylaminopurine (1 $mgdm^{-3}$) were filter sterilized after autoclaving.

Component	Concentration ($mgdm^{-3}$)
Murashige and Skoog basal salt mixture	4400
Myoinositol	100
Sucrose	3000
Glutamine	200[Filter sterilised]
BAP	1.0[Filter sterilised]
H-Vitamin	10 cm^3/dm^3

Table 2.1 Oak embryo maintenance medium

Component	Concentration ($mg100cm^{-3}$)
Nicotinic acid	50
Thiamine	5
Pyridoxine-HCl	5
Glycine	20

Table 2.2 H-vitamin stock solution

2.2.2.3 Sitka Spruce embryogenesis (*Picea sitchensis* (Bong.) Carr.) initiation medium

Solid and suspension embryonal suspensor masses of Sitka Spruce were maintained on Sitka Spruce embryo initiation medium (Gupta and Durzan, 1986). The components of this medium are outlined in Table 2.3.

The medium was adjusted to pH 5.7 for solid medium and pH 5.0 for liquid medium.

Component	Concentration $mgdm^{-3}$
KNO_3	2034.0
$CaCl_2$	220.0
$CoCl_2.6H_2O$	0.025
FeNaEDTA	36.7
KI	8.0
$Na_2Mo.H_2O$	0.25
Myoinositol	1000.0
2,4-D	1.1
NH_4NO_3	206.0
KH_2PO_4	85.0
$MgSO_4$	185.0
$CaSO_4.5H_2O$	0.025
H_3BO_3	6.2
$MnSO_4.H_2O$	17.0
$ZnSO_4.7H_2O$	86.0
Casamino acid	500.0
Kinetin	0.4
Sucrose	30000.0
Glutamine	450.0
BAP	0.4
H-Vitamins	10 cm^3/dm^3

Table 2.3. Sitka Spruce embryo initiation medium

2.2.2.4 Oak (*Quercus robur* L.) maturation medium

All Oak embryo maturation media were based on variations of Murashige and Skoog medium (Table 2.1) and Woody Plant Medium (WPM) (Lloyd and McCown, 1980) (Table 2.4, 2.5) with the exception of P24 maturation medium (Wilhelm, E., Austrian Research Centre (per comm)) (Table 2.6, 2.7, 2.8, 2.9). A total of 7 experiments were carried out each utilising variations of the above media.

Component	Concentration $mgdm^{-3}$
Sucrose	20000
Gelrite	3000
SCW	100 cm^3/dm^3

Table 2.4 Woody Plant Medium

To the above 100 cm^3 of SCW was added from a stock solution outlined in Table 2.5 below. Originally one litre of distilled water was placed in a 2 dm^3 conical flask and all of the below ingredients were added. WPM medium was prepared in 2 dm^3 quantities. The second litre of distilled water was then added. K_2SO_4 was dissolved in 500 cm^3 of distilled water before it was added to the stock. The stock was stored in 100 cm^3 quantities in plastic containers at – 4°C. SCW stock was stored in 10 cm^3 quantities in plastic containers at – 4°C.

Component	Concentration $mgdm^{-3}$
NH_4NO_3	2000
$Ca(NO_3)_2.4H_20$	2250
K_2SO_4	5950
$CaCl_2.2H_20$	480
KH_2PO_4	870
H_3BO_3	31
$NaMO.O_4.2H_2O$	1.25
$MgSO_4.7H_2O$	1750
$MnSO_4.7H_2O$	112
$ZnSO_4.7H_2O$	43
$CuSO.5H_2O$	1.25
Thiamine-HCl	5
Nicotinic acid	2.5
Pyridoxine-HCl	2.5
Glycine	10
Myoinositol	500
Fe 330	200

Table 2.5 SCW stock solution

Component	Volume/weight per dm^3
Sucrose	30000 mg
P24 macro nutrients(5X)	200 cm^3
P24 micro nutrients (100X)	10 cm^3
Myoinositol (55 mM)	10 cm^3
Nicotinic acid (8 mM)	0.5 cm^3
Thiamine-HCl (3 mM)	0.1 cm^3
Pyridoxine-HCl (0.5 mM)	5 cm^3
Fe NaEDTA (10 mM)	11 cm^3
$FeCl_3$ (10 mM)	5 cm^3
Arginine-HCl	500 mg
Difco Noble Agar	8000 mg
pH = 5.7	Autoclave 25 min

Table 2.6. P24 Medium

Component	Concentration ($mgdm^{-3}$)
$Ca(NO_3)_2.4H_2O$	6610.0
$Mg(NO_3)_2.6H_2O$	1920.0
KH_2PO_4	4080.0
K_2SO_4	4360.0
$NH_4H_2PO_4$	1440.0
KNO_3	2530.0
NaCl	58.0
Storage at +4°C	

Table 2.7. P24 Macro Nutrients (5X) Stock

Component	Concentration (cm^3100cm^{-3})
KI (60 mM)	0.6
$CuSO_4.5H_2O$ (4 mM)	25.0
$ZnSO_4.7H_2O$ (20 mM)	20.0
$COCl_2.6H_2O$ (4mM)	0.5
$Na_2Mn.O_4$ (4 mM)	0.5
$NiCl_2.6H_2O$ (1mM)	2.0
$MnSO_4.H_2O$ (20 mM)	10.0
H_3BO_3 (50 mM)	24.0
Bring up to 100 cm^3 with dH_2O	Store at +4°C

Table 2.8. P24 Micro Nutrients (100X) Stock

Medium Variant	Agar %	Sucrose %	AC %	ABA µM
1	0.8	3	1	
2	0.8	5		
3	1.0	3		
4	1.0	5		
5	0.8	3		
6	0.8	3		2.5

Table 2.9 P24 Maturation medium

Agar, sucrose and activated charcoal were added prior to autoclaving. Abscisic acid was filter sterilized into the autoclaved medium.

2.2.2.5 Sitka Spruce (*Picea sitchensis* (Bong.) Carr.) embryo maturation medium

Sitka Spruce ESMs were matured on Sitka Spruce embryo development medium (Table 2.10) and Sitka Spruce embryo germination medium (Table 2.11) (Gupta and Pullman, 1991).

Component	Concentration $mgdm^{-3}$
As for embryo initiation medium except	
KNO_3	1170.0
Arginine	40.0
Asparagine	100.0
Sucrose	30000.0
Activated charcoal	1025.0

Table 2.10. Sitka Spruce embryo development medium

The autoclaved medium was placed in a water bath at 42°C, ABA (50 $mgdm^{-3}$) which had been dissolved in a few drops of NaOH was filter sterilised into the autoclaved medium. The medium was incubated at this temperature for a futher 10 min with swirling every 2-3 min.

Component	Concentration $mgdm^{-3}$
NH_4NO_3	360.0
$Ca(NO_3)_2.4H_2O$	709.0
KNO_3	506.0
KH_2PO_4	272.0
$MgSO_4.7H_2O$	493.0
KCl	149.0
Gelrite	7000.0
Sucrose	30000.0

Table 2.11. Sitka Spruce somatic embryogenesis, embryo germination medium

2.2.3 Growth Conditions

All cultures were grown at 24°C ± 2°C in a constant temperature growth room. Embryogenic suspension cultures were grown on a gyratory shaker at 100 r.p.m under warm white fluorescent lights using a 16 h photoperiod light intensity with approximately 20μmoles/m^2/s of photosynthetically active radiation. Embryogenic solid cultures were grown in the dark in boxes.

2.2.4 Growth of Plant Material

Subculturing of Oak and Sitka Spruce embryogenic callus cultures was performed in the laminar airflow cabinet with sterilized instruments. Embryogenic solid medium cultures were grown on 20 cm^3 of embryogenesis medium in 9 cm petri dishes. Subculturing of solid media cultures involved removal of necrotic tissue, which was black or brown in appearance. Each piece of callus was then split into approximately 5 pieces. Callus pieces were then placed onto a dish of fresh medium. Petri dishes were sealed with a layer of parafilm, labelled clearly with a permanent marker and placed in the growth room. Oak solid embryogenic cultures were subcultured every 4 weeks and Sitka Spruce embryogenic solid cultures subcultured every 2-3 weeks. To decrease the chance of loss of stocks all cultures were not subcultured onto the same batch of medium as the medium may have become contaminated at some stage during its preparation or some ingredient may have been omitted.

Suspension cultures were grown in 50 cm^3 of embryogenesis medium in sterile Erlenmeyer flasks. Subculturing involved transfer of 50 cm^3 of fresh medium into the flask of cells and conditioned medium. The neck of the flask was flamed for a few seconds before media was poured from them. The resulting 100 cm^3 of medium was

then split into 2 x 50 cm^3 quantities ensuring that each flask was housing approximately half the suspension cells from the original flask. Subculturing was performed every 10-12d.

Oak and Sitka Spruce single cell cultures were grown in pre-sterilized 24 well multi-well plates. Each well contained 1 cm^3 of cells and medium. Plates were sealed with a layer of parafilm and placed on the gyratory shaker at 100 r.p.m.

2.2.5 Production of Oak and Sitka Spruce Single Cells

2.2.5.1 Oak single cell cultures

Oak embryogenic suspension callus cultures were filtered through a 100 micron mesh and the resulting retenate was transferred to MS suspension medium containing 0.01 $mgdm^{-3}$ BAP. The concentration of BAP was thus reduced 100-fold from the normal concentration (1$mgdm^{-3}$). Culture aggregation was related to cytokinin concentration and under the conditions of low cytokinin, the embryogenic suspension callus became friable and single cells dissociated from them.

2.2.5.2 Sitka Spruce single cell cultures

Sitka Spruce embryo cultures released single cells in extremely low numbers even under conditions of low cytokinin. It was necessary therefore to actively macerate the cultures. A range of protocols for maceration were examined utilizing pectinase digestion of cells.

Sitka Spruce ESMs were incubated in a range of pectinase concentrations (0-5%) in embryogenesis medium for a range of time intervals. Separation of the cells from embryogenic heads from those cells of the suspensors was achieved on a Percoll gradient.

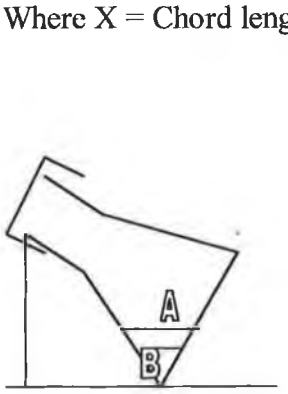
2.3 Analytical Methods

2.3.1 Measurement of Oak and Sitka Spruce Suspension Cultures

Growth of both Oak and Sitka Spruce embryonal suspensor cultures was assessed by recording the settled cell volume of the flasks (Gilissen *et al.*, 1983). Settled cell volumes were recorded by placing flasks on a stand at a 45° angle. Flasks were left to settle for 15 min to allow cells to settle to the bottom of the flasks. With a 12 inch plastic transparent ruler the chord length of settled cells from one end of the base of the flask to the other was measured in millimetres. Settled cell volume was then converted to cm^3 using the following equation:

$$\mathbf{SCV\ (cm^3) = 10\text{^}((X \times 0.0378) - 1.3489)}$$

Where X = Chord length



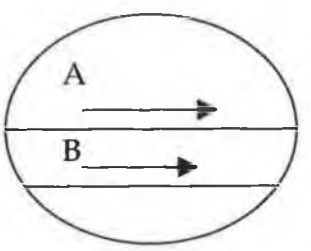


Figure 2.1

Diagram showing base of a 250 cm^{-3} Erlenmeyer flask. Chord A is length of settled 50 cm^{-3} embryogenesis medium. Chord length B is chord length of settled suspension cells recorded in millimetres and designated X in the equation.

2.3.2 Measurement of Growth of Oak and Sitka Spruce Single Cells.

In order to monitor the growth of isolated single cells in culture, it was necessary to use a device that could measure the growth in small volumes (1-2 cm^3) with a high accuracy and precision. This was achieved using a 24 well multi-well plate reader. In brief, light from an ultra-bright green light emitting diode (LED) (λmax 565nm) positioned over a well of the plate was passed through the well and the transmitted light detected with a light dependent resistor (LDR) immediately below the well. The resistance change in the LDR was converted to a voltage output using a voltage divider circuit and this fed into a PC through a data acquisition card. This combination of LED, LDR and the voltage driver circuit resulted in a sensor of culture absorbance. This sensor was replicated 24 times to produce a sensor array which could monitor each well of the plate. The linearity of voltage output with absorbance was excellent for the device ($r^2 = 0.9974$) and the resolution was 0.0014A (corresponding to 50 $mgdm^{-3}$ fresh weight Sitka Spruce embryo culture). The voltage was converted to absorbance at 565 nm.

2.3.3 Tri parental mating techniques

2.3.3.1 Gene cloning

The choice of vector system for a particular transformation procedure depends on many factors, not least of which is the aim of the experiment and the particular species involved. Binary vectores are generally easily isolated from E. *coli*. Thus all recombinant DNA manipulations may be carried out in E. *coli*. Mobilization of such vectors into *Agrobacterium* is generally not difficult. Apart from mobilization efficiency, the frequency of transconjugant selection in *Agrobacterium* is much higher for binary

vectors as co-integration is not required. The transformation experments carried out in this thesis utilized a variant of the pBin19 *Agrobacterium* binary plasmid vector, pBI121.

2.3.3.2 Strains used in tri parental mating

E. *coli* donor strain Jm83::pBIN19. This strain is kanamycin resistant and it contains the cloning vector.

E.coli helper strain HB101::pROK2013.

XL1::JIT6035SGUS Gus containing plasmid

Agrobacterium tumefaciens recipient strain LBA4404. This was resistant to rifampicin antibiotic.

E.coli (Jm83::pBIN19 and HB101::pROK2013) strains were cultured on Nutrient agar slopes at 30°C containing 25 $mgdm^{-3}$ kanamycin sulfate (filter sterilized into agar). An overnight culture was prepared in Nutrient broth containing 25 $mgdm^{-3}$ kanamycin sulfate at 30°C prior to mating. *Agrobacterium tumefaciens* LBA4404 strain was cultured on nutrient agar plates at 30°C for 24h. Nutrient broth was inoculated and cultured overnight at 30°C in preparation for mating.

In this protocol the E .*coli* strain Jm83::pBIN19 was used as the recipient in the first mating with an *E.coli* strain harboring pRK2013, a helper plasmid acting as a donor strain. In this mating pRK2013 was transferred to the strain harboring pBIN19. The second mating involved mobilizing pBIN19 by the pRK2013 helper plasmid to *Agrobacterium* LBA4404 which is the final recipient in the mating.

2.3.3.3 Tri parental mating protocol

From overnight cultures 0.7 cm^3 of donor (pRK2013) and 0.7 cm^3 of recipient (Jm83::pBIN19) were placed in a sterile microcentrifuge tube and centrifuged at 13,000 x *g* for 5min. The supernatant was decanted and the pellet resuspended in 200 µl of

Nutrient broth without antibiotics. This was then transferred as a single drop onto the surface of a Nutrient agar plate again containing no antibiotics and cultured at 30°C overnight. The next day the plate was flooded with 4 cm^3 of Nutrient broth, cells were mixed in the broth and removed with a pipette to a microfuge tube (Eppendorf). This culture then acted as the donor for the second mating which was a repeat of the above procedure with LBA4404 acting as the recipient strain in this case. When the mating was complete 0.1 cm^3 was spread plated on Nutrient agar containing 25 $mgdm^{-3}$ kanamycin sulfate and 100 $mgdm^{-3}$ rifampicin. The plates were then cultured at 30°C overnight and examined the next day for colonies indicating a successful mating.

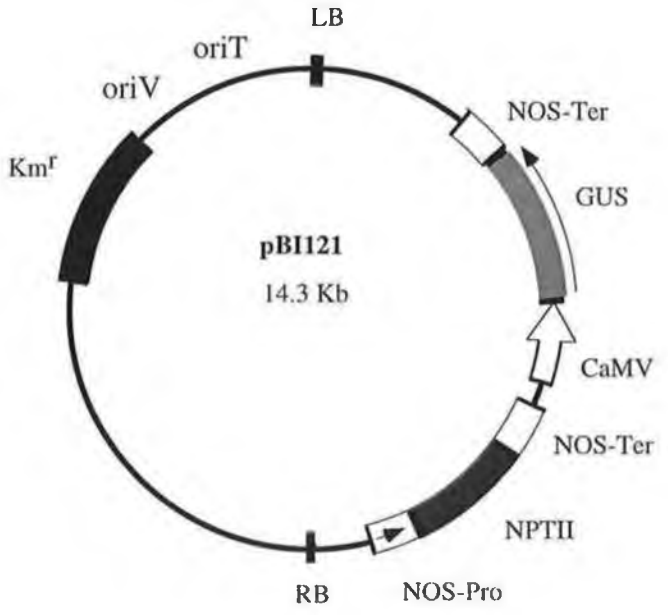


Figure 2.2

Circular map of plasmid pBI121 (Jefferson *et al*, 1987). Abbreviations: LB and RB, left border and right border of T-DNA region, respectively; OriT, ColEI origin of replication; OriV, broad host-range origin of replication; Km^r, kanamycin resistance gene for selection in bacteria; NOS-Pro, nopaline synthase promoter; NPTII, neomycin phosphotransferase gene; NOS-Ter, nopaline synthase polyadenylation site; CaMV, cauliflower mosaic virus (CaMV) 35S promoter; GUS, β -glucuronidase gene.

2.3.4 Growth of *Agrobacterium* Strains

A supervirulent strain of *Agrobacterium tumefaciens* LBA4404::pBI121 was grown on Nutrient agar plates (0.25 $mgdm^{-3}$ kanamycin, 0.25 $mgdm^{-3}$ rifampicin) at 30°C in the dark. At temperatures above 30°C there was a risk of the *Agrobacterium* losing its plasmid. Nutrient broth cultures were initiated from Nutrient agar plates. *Agrobacterium tumefaciens* LBA4404 does not contain the kanamycin resistance gene (*npt* II) and therefore is simply grown on Nutrient agar plates plus 0.25 $mgdm^{-3}$ rifampicin at 30°C in the dark.

2.3.5 *Agrobacterium*-mediated Transformation Experiments

Overnight broth cultures of LBA4404::pBI121 grown to an OD_{600} of 0.8-1.1 (1-3 x 10^9 bacteria cm^{-3}) were used in all transformation experiments unless otherwise stated. A number of experimental parameters were altered throughout the range of transformation experiments. Parameters altered included dilution of *Agrobacterium* strain. All dilutions were prepared in conditioned Oak or Sitka Spruce embryogenesis medium and dilutions ranged from 10%-100%. Incubation time was defined as the period of time cells were exposed to the *Agrobacterium* dilutions. Incubation times varied from 10min-420min. Co-cultivation time was defined as the time cells were grown in medium with residual *Agrobacterium* after cells have been removed from the *Agrobacterium* dilution and before cefotaxime was added to inhibit further growth of *Agrobacterium*. Co-cultivation times varied from 24h-96h. Acetosyringone concentrations varied from 0μM–125μM. Acetosyringone was added from a 20 mM stock in methanol to the Nutrient broth for *Agrobacterium* culture. Cell quantities also varied throughout the range of transformation experiments.

All transformation experiments were performed in the same manner. A quantity of cells was sieved through a sterile 100 μm sieve from 7d old flasks of Oak or Sitka Spruce embryogenic suspension cells which were in their mid exponential phase. A known quantity of cells was weighed out and transferred to the *Agrobacterium* dilutions. Following the incubation period cells were again sieved through 100 μm sieves or centrifuged at 4,000 x *g* for 10 min and washed in sterile distilled water. Cells were then transferred to medium minus antibiotics in 250 cm^3 Erlenmeyer flasks and co-cultivated with the residual *Agrobacterium* for a specified time. Flasks were placed in the growth room on a gyratory shaker at 100 r.p.m. Cells were assayed for the continued presence of the *GUS* gene periodically.

All control transformations were carried out with *Agrobacterium* strain LBA4404.

2.3.6 Histochemical Assay of β-glucuronidase Activity

Cells were weighed and fixed in 1 cm^3 of formaldehyde (0.3% formaldehyde, 0.3M mannitol, 10 mM MES in 30 cm^3 dH_2O) for 1h in 1.5 cm^3 microfuge tubes (Eppendorf) (Jefferson, 1987). To 1 cm^3 of 50 mM $NaPO_4$ (pH 7.0), 1 cm^3 X-gluc (5-bromo-4-chloro-3-indoyl β-D-glucuronic acid) and 10 μl dimethylformamide was added. Each sample was incubated overnight at 37°C in the above in a 1.5 cm^3 microfuge tube (Eppendorf). Samples were analyzed for the number of densely blue stained areas per gram of cells. Staining indicated β-glucuronidase activity.

2.3.7 Continued Growth of Oak and Sitka Spruce Transformed Cells

Following the specified co-cultivation time, 500 $mgdm^{-3}$ cefotaxime was added to transformed cultures to eliminate *Agrobacterium*. Three days later 3 $mgdm^{-3}$ paromomycin was added to Sitka Spruce transformed cultures and 30 $mgdm^{-3}$ paromomycin was added to transformed Oak cultures. Levels of cefotaxime and paromomycin were maintained in all subsequent subcultures.

2.3.8 Microscopy

Microscopy and image capture of Oak and Sitka Spruce embryogenic suspension cultures and single cell cultures was carried out using a CCD type colour video camera (JVC model tyoe KY-F55B) attached to an Olympus BX40 bight field microscope by a C-mounted adaptor. Images were captured using the OPTIMAS 6 image analysis programme. Approximately 100-200 µl of cell suspension were placed on a microscope slide and a cover slip placed on top. Cell samples were viewed under 10 X magnification on a compound microscope. Images were digitised and could be visualised on the computer screen and could be saved in a variety of file formats.

2.3.9 Statistical Analysis

Statistical analysis of data was performed using the SPSS statistical package for all experiments. Means, standard error of means, One way ANOVAs and Tukey's tests were used throughout.

Chapter 3

Growth of Oak (*Quercus robur* L.) Embryogenic Suspension cultures and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Embryonal Suspensor Mass in Liquid Culture and Determination of Selective Medium for *Agrobacterium*-mediated Transformation.

3.1 Introduction

The genetic transformation of woody plants using *Agrobacterium* relies on a number of factors including the selection and regeneration of transformed cells and the elimination of the bacterium from the *in vitro* environment. For transformation the Oak embryogenic suspensor cells and the Sitka Spruce embryonal suspensor masses are infected by co-cultivation with the disarmed *A. tumefaciens* in an antibiotic free environment for up to 3d. Following this co-cultivation period the *Agrobacterium* needs to be suppressed so as not to interfere with the growth and development of the transformed plant cells. This is usually carried out by the transfer of the plant cells to a selective medium which contains *Agrobacterium*-suppressing antibiotics (Horsch *et al.*, 1985). As the plant tissue in culture is affected by the different components in its culture medium it is important to use antibiotics which have a negligible effect on plant growth and regeneration. Carbenicillin and cefotaxime, both belonging to the β-lactam group, are the two antibiotics most widely employed to eliminate *Agrobacterium* from culture (Shaw *et al.*, 1983; Mathias and Boyd, 1996). Cefotaxime was found to be the most effective against *A. tumefaciens* LBA4404 (Shacleford and Chlan, 1996). Antibiotics are widely used in the selection of transformed plant cells. The choice of selection antibiotics used is based on the antibiotic resistance gene present in the *A. tumefaciens* strain. Selection antibiotics most commonly used in the presence of the *npt* II gene are kanamycin, neomycin and paromomycin.

The following set of experiments were performed to assess the growth of both Oak (*Quercus robur* L.) embryogenic suspensor cells and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses firstly in antibiotic free liquid culture medium. Secondly we needed to evaluate whether or not both cell types would grow uninhibited in the

presence of cefotaxime antibiotic at varying concentrations in the liquid culture medium. Finally we needed to observe the growth of both cell types in liquid culture medium containing both kanamycin and paromomycin antibiotics.

The following results outline the growth cycles of both Oak embryogenic suspension cultures and Sitka Spruce embryonal suspensor mass suspension cultures in the presence and absence of antibiotics utilised in the *Agrobacterium*-mediated transformation protocols outlined in the next chapter.

3.2 Results

3.2.1 Growth of *Agrobacterium tumefaciens* LBA4404::pBI121 in Cefotaxime Antibiotic.

Agrobacterium strain LBA4404::pBI121 was grown in 20 cm^3 Nutrient broth plus 0 $mgdm^{-3}$, 100 $mgdm^{-3}$, 300 $mgdm^{-3}$, 500 $mgdm^{-3}$, 700 $mgdm^{-3}$, 1000 $mgdm^{-3}$, 1500 $mgdm^{-3}$, 2000 $mgdm^{-3}$ cefotaxime antibiotic at 30°C on a gyratory shaker at 100 r.p.m. Following a 24h culture period 3 cm^3 samples were aseptically taken and their optical densities at 600nm were read in a spectrophotometer. *Agrobacterium* cultures were returned to the growth room for a further 24h and their optical densities were read again after a total 48h culture period.

Figure 3.1 shows growth of *Agrobacterium tumefaciens* LBA4404::pBI121 over a 48h period. Results show that *Agrobacterium* failed to grow in all cefotaxime concentrations but grew rapidly in Nutrient broth in the absence of cefotaxime.

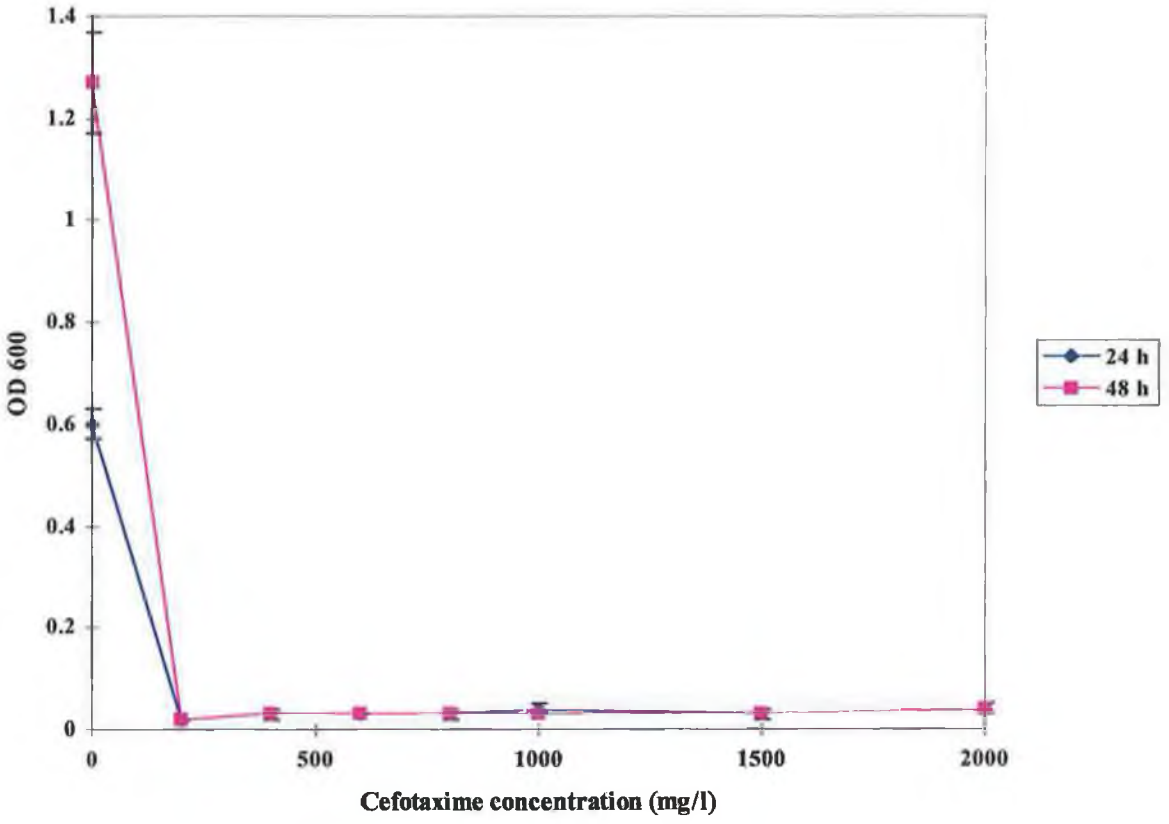


Figure 3.1

Growth of *Agrobacterium tumefaciens* LBA4404::pBI121 in concentrations of cefotaxime antibiotic ranging from 0 $mgdm^{-3}$-2000 $mgdm^{-3}$ over 48 h in Nutrient broth. The OD_{600} of 3 cm^3 samples were read on a spectrophotometer. The experiment was carried out in duplicate and standard errors of the mean are included. The units $mgdm^{-3}$ are consistent with mg/l.

3.2.2 Growth of Oak (*Quercus robur* L.) embryogenic suspension cultures

Growth of Oak embryogenic suspension cultures was investigated. Oak embryogenic suspension cell growth curves were required to evaluate the optimum time for subculture, to maintain embryogenic potential and for the reduction of cell death. It was vital to later experiments on cell growth in the presence of antibiotics to have a control growth curve to compare growth in the absence of any additional media components.

On the first day of subculture a total of 10 flasks were set aside for assessment of cell growth. The settled cell volume of each flask was measured and the results recorded as 0d growth. Flasks were taken from the culture room every 2d and settled cell volumes measured over a total of 14d. This experiment was repeated on a new set of 10 flasks. The results of these growth cycles are presented below.

Figure 3.2 shows a 14d growth cycle of Oak embryogenic suspensor cells. The initiating settled cell volume was recorded as 0.75 cm^3. Table 3.1 outlines the increase in settled cell volume from 0d-14d. From the graph it can be seen that growth of embryogenic suspensor cells starts to plateau between 12d and 14d. The graph shows that the slope of the line decreases from 4d and therefore after this there is a constantly decreasing growth rate. Table 3.1 also shows the cell doubling time and the number of cell doublings in the growth cycle.

The number of doublings was calculated by the following equation:

$$\textbf{Doublings} = \textit{increase in } \log_{10} \textit{ settled cell volume} \div \log_{10} 2$$

The doubling time was calculated by the following equation:

Doubling Time = ***Time frame of cell growth (d) ÷ No. of cell doublings***

Results were such that a clear picture of Oak embryogenic suspension cell growth was evident. The optimum subculture time was evaluated at 10d to ensure maintenance of culture embryogenic potential and avoidance of necrotic tissue formation. Experiments on the growth of suspension cultures in media with the addition of supplementary ingredients such as antibiotics could now be examined.

Time	Increase in log settled cell volume	No. of Doublings	Doubling Time (d)
0d-4d	0.54	1.79	2.23
4d-12d	0.38	1.26	6.35
12d-14d	0	0	∞

Table 3.1 Doubling time and no. of doublings in a 14d growth cycle of Oak *(Quercus robur* L.) embryogenic suspensor cells.

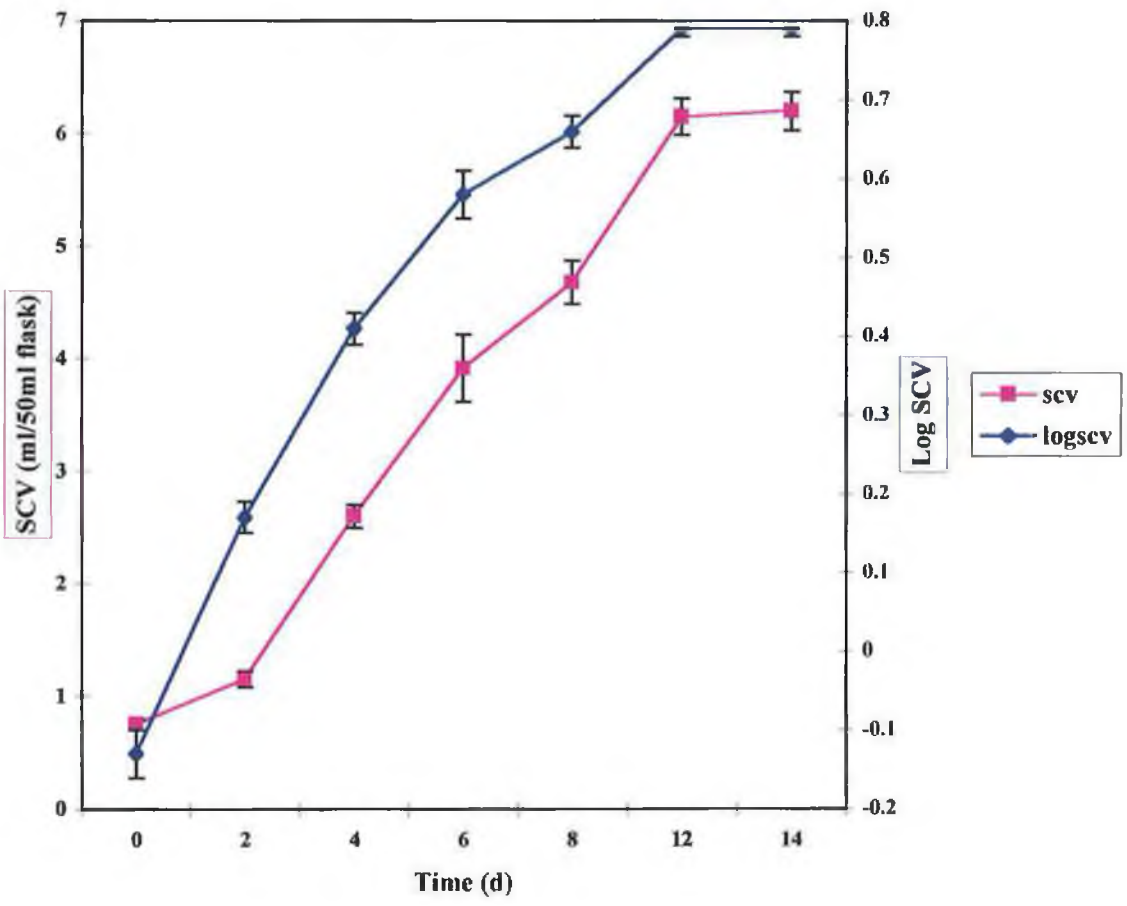


Figure 3.2

Growth of Oak (*Quercus robur* L.) embryogenic suspension cultures over time. Results are the average of 10 flasks containing 50 ml of medium per flask. The experiment was carried out in duplicate and standard errors of the mean are included.

3.2.3 Growth of Oak (*Quercus robur* L.) embryogenic suspension cultures in varying concentrations of cefotaxime antibiotic

The bactericidal antibiotic cefotaxime is added to cultures following *Agrobacterium*-mediated transformation. However, it is important that it does not also strongly affect growth of the transformed cultures. Cell growth is essential for selection and regeneration and disruption of the cell growth cycle would therefore not allow for the use of cefotaxime as an *Agrobacterium* inhibitor in transformed cultures of Oak embryogenic suspension cells.

Cefotaxime concentrations ranging from 0 $mgdm^{-3}$-1000 $mgdm^{-3}$ were filter sterilised into autoclaved Murashige and Skoog medium prior to subculture of suspensions. Medium was aseptically removed from 10d old Oak suspension cultures which were subsequently subcultured with the cefotaxime containing medium. Five flasks were sampled for each cefotaxime concentration. All flasks were placed in the culture room on the shaker (100 r.p.m). Settled cell volumes were routinely recorded every 2d for a 14d growth period. The experiment was repeated following completion of the original experiment. Results are presented below.

Figure 3.3 shows the growth of Oak embryogenic suspensor cells in concentrations of cefotaxime antibiotic varying from 0 $mgdm^{-3}$–1000 $mgdm^{-3}$. Starting inocula vary from 0.49 cm^3–1.89 cm^3. From the graph it was evident that at 12d most concentrations were still growing or had just started to level off.

Results show that concentrations of cefotaxime antibiotic up to 1000 $mgdm^{-3}$ did not inhibit Oak embryogenic cell growth. However ANOVA results show that the effects of cefotaxime concentration were highly significant over time ($p<0.05$). Table 3.2 outlines the

doubling times and number of doublings in all growth cycles for different time frames. With the exception of 0 $mgdm^{-3}$ cefotaxime in all other concentrations growth between 12d-14d appeared to level off with doubling times showing a very high increase. All concentrations showed the greatest growth phase from 0d-8d with doubling times averaging between 3d-4d. The effects of cefotaxime concentration on the doubling time of cultures from 4d-12d are outlined in Fig. 3.4. Doubling times decrease at 300 $mgdm^{-3}$-500 $mgdm^{-3}$ cefotaxime concentration and rise again thereafter to doubling times similar to those for 0 $mgdm^{-3}$. Tukey's HSD test shows that doubling times for 300 $mgdm^{-3}$ and 500 $mgdm^{-3}$ cefotaxime are significantly different to all other doubling times.

As results suggest the continued growth of Oak embryogenic suspension cultures in the presence of cefotaxime antibiotic at concentrations as high as 1000 $mgdm^{-3}$ we can safely utilise this antibiotic for the inhibition of *Agrobacterium* from transformed cultures.

With the identification of an antibiotic for the suppression of *Agrobacterium* growth the problem of selection of transformed cells as opposed to non-transformed cells from culture presented itself. The following set of experiments deals with the identification of an antibiotic for this purpose.

[Cefotaxime] $mgdm^{-3}$	Time from start	Log increase in settled cell volume	No. SCV doublings in the time frame	Doubling Time (d)
0	0d-8d	0.73	2.43	3.30
0	8d-12d	0.92	1.03	3.88
0	12d-14d	0.27	0.89	2.23
100	0d-8d	0.59	1.96	4.08
100	8d-12d	0.38	1.26	3.11
100	12d-14d	0.01	0.03	60.24
300	0d-8d	0.67	2.23	3.59
300	8d-12d	0.5	1.66	2.41
300	12d-14d	0	0	∞
500	0d-8d	00.71	2.36	3.39
500	8d-12d	0.48	1.59	2.51
500	12d-14d	0.04	0.13	15.06
700	0d-8d	0.69	2.29	3.49
700	8d-12d	0.18	0.59	6.69
700	12d-14d	-0.01	-0.03	-60.24
900	0d-8d	0.82	2.72	2.99
900	8d-12d	0.31	1.03	3.89
900	12d-14d	0.09	0.29	6.69
1000	0d-8d	0.82	2.72	2.93
1000	8d-12d	0.25	0.83	4.82
1000	12d-14d	0.01	0.03	60.24

Table 3.2 Doubling time (d) and No. of doublings of Oak (*Quercus robur* L.) embryogenic suspension cells in varying concentrations of cefotaxime antibiotic in a 14d cell growth cycle.

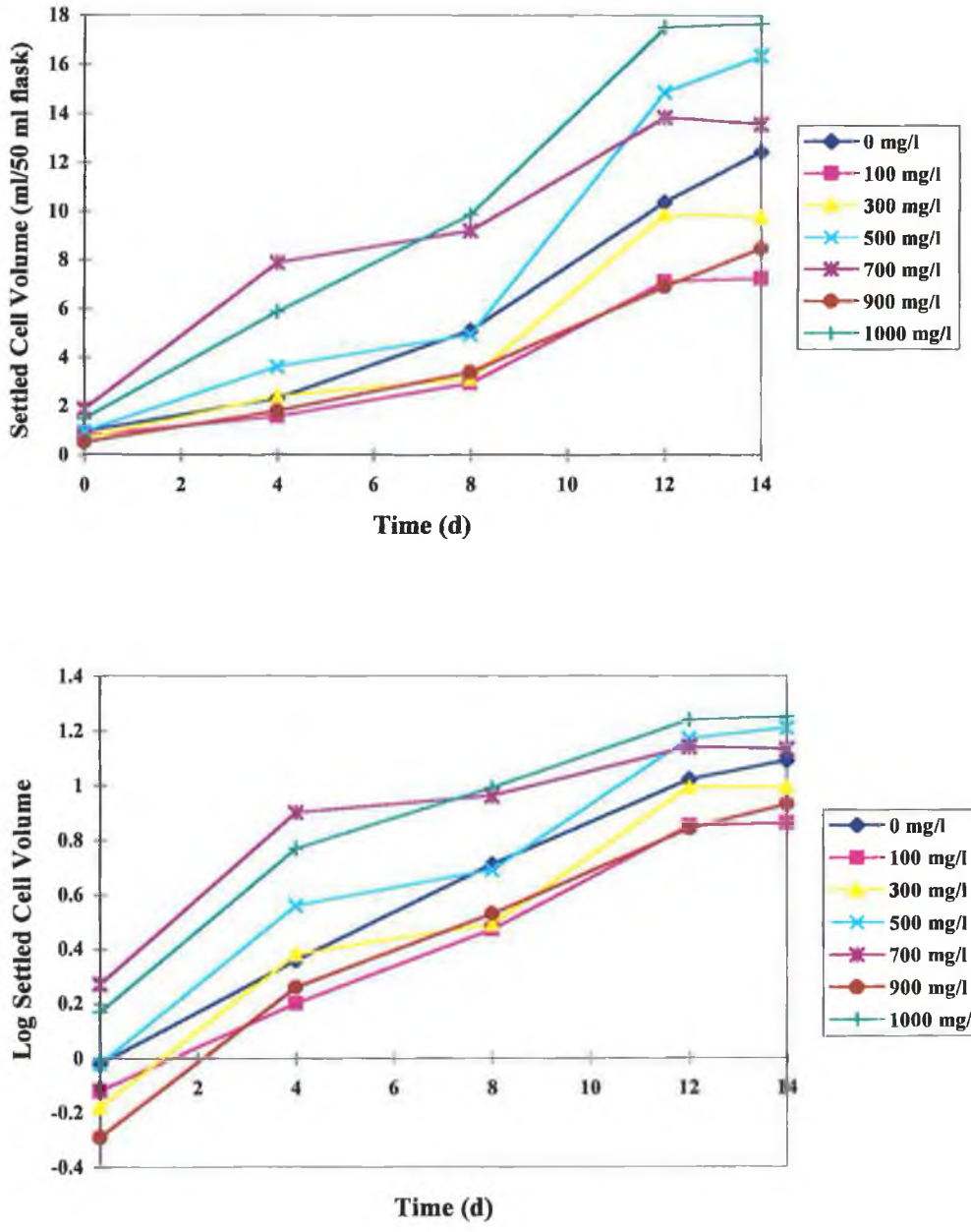
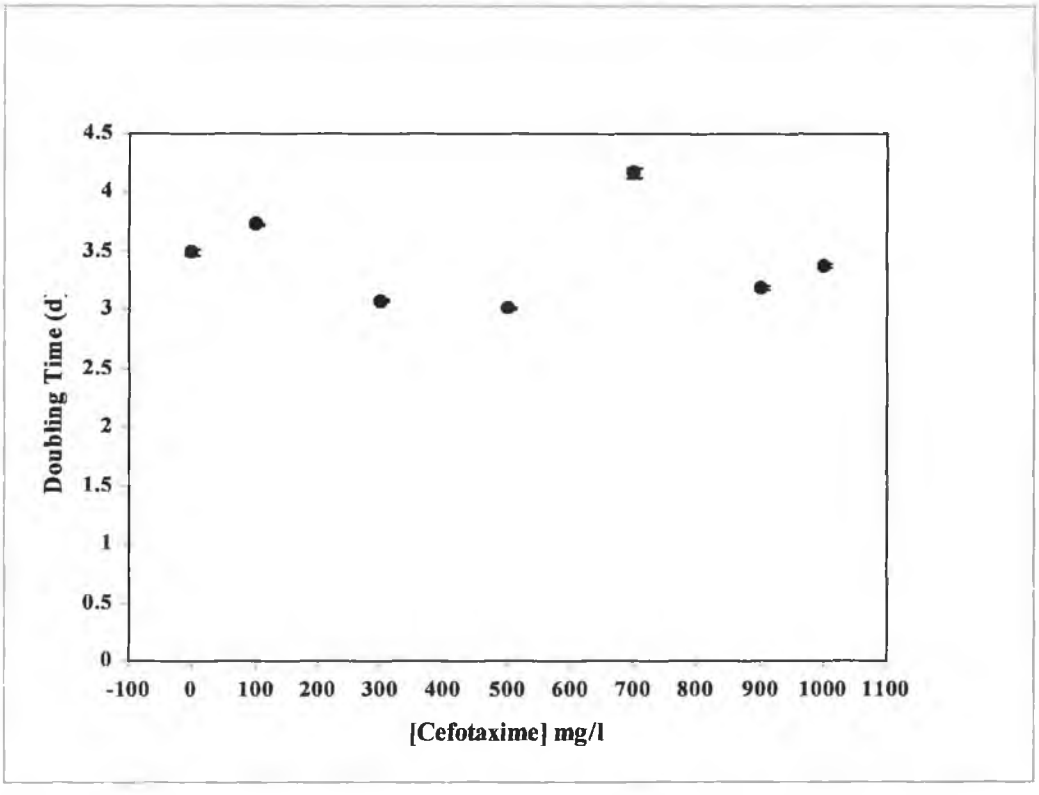


Figure 3.3

Growth of Oak (*Quercus robur* L.) embryogenic suspension cultures in different concentrations of cefotaxime antibiotic over time. Results were recorded from the average of 5 flasks per cefotaxime concentration each containing 50 cm^{-3} medium per flask . The experiments were carried out in duplicate. Repeat experiments did not differ significantly. Since error bars overlapped they were omitted from the graph. The units $mgdm^{-3}$ are consistent with mg/l.



DT12

Tukey HSD[a]

CEF	N	Subset for alpha = .05					
		1	2	3	4	5	6
500.00	10	3.0205					
300.00	10	3.0776					
900.00	10		3.1929				
1000.00	10			3.3783			
.00	10				3.4877		
100.00	10					3.7340	
700.00	10						4.1716
Sig.		.469	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Figure 3.4

Mean doubling time (d) of Oak embryogenic suspension cultures from day 4 to day 12 in different concentrations of cefotaxime antibiotic. Experiments were carried out in duplicate. Repeat experiments did not differ significantly and standard errors of the mean are included. Results of Tukey's HSD test are outlined in the table, doubling times in the same column are significantly different from doubling times in other columns but not from each other.

3.2.4 Growth of Oak (*Quercus robur* L.) embryogenic suspension cultures in varying concentrations of kanamycin antibiotic

Agrobacterium-mediated transformation of Oak embryogenic suspensor cells results in the incorporation of an antibiotic resistance gene into the plant genome. In the case of our *Agrobacterium* strain LBA4404::pBI121 the gene was *npt* II which confers resistance to a number of aminoglycoside antibiotics such as kanamycin, paromomycin and neomycin. The following experiments investigate the growth of Oak embryogenic suspension cultures in the presence of the kanamycin antibiotic. Untransformed cells *i.e.* cells which do not contain the *npt* II gene should not grow successfully in the presence of kanamycin in their growth medium. Continued growth of untransformed cells hinders the identification of transformed cells as they too will grow in the presence of kanamycin.

Kanamycin concentrations varying from 0 $mgdm^{-3}$-1000 $mgdm^{-3}$ were filter sterilised into autoclaved Oak embryogenesis medium. Flasks were prepared as with the cefotaxime experiment and again 5 flasks were sampled for each kanamycin concentration. Flasks were removed from the growth room every 2d and settled cell volumes recorded for a 14d growth period. The experiment was repeated following completion of the original experiment. The results of the growth of Oak embryogenic suspension cells in kanamycin antibiotic are presented below.

Figure 3.5 shows the graph of Oak embryonal suspension cell growth in concentrations of kanamycin antibiotic varying from 0 $mgdm^{-3}$–1000 $mgdm^{-3}$. The graph outlines the continued growth of cells in all concentrations of kanamycin in a 14d growth cycle. Initiating settled cell volumes varied from 0.67 cm^3–1.68 cm^3. Table 3.3 shows the recorded doubling times and number of cell doublings for each concentration of kanamycin.

The highest growth rate was from 0d-8d with doubling times ranging from 3-5d. ANOVA results showed that the effects of kanamycin concentration and time were both highly significant ($p < 0.05$). The effect of kanamycin concentration on the doubling time of cells is outlined in Fig. 3.6. The graph shows that doubling time increases at 100 $mgdm^{-3}$ and decreases again. At 700 $mgdm^{-3}$ kanamycin the doubling time decreases significantly and this suggests that kanamycin may even be promoting cell growth at high concentrations. Tukey's HSD test shows that doubling times recorded for 700 $mgdm^{-3}$ and 100 $mgdm^{-3}$ kanamycin are significantly different from all other doubling times.

The continued growth of Oak embryogenic suspension cultures in concentrations of kanamycin as high as 1000 $mgdm^{-3}$ suggests that it is unsuitable as an antibiotic for the selection of transformed cells. An alternative antibiotic needed to be found and the next experiment investigates paromomycin as that alternative.

[Kanamycin] $mgdm^{-3}$	Time from start	Log increase in settled cell volume	No. SCV doublings in the time frame	Doubling Time (d)
0	0d-8d	0.51	1.69	4.72
0	8d-12d	0	0	∞
0	12-14d	0.08	0.27	7.53
100	0d-8d	1.04	3.45	2.31
100	8d-12d	0.09	0.29	13.38
100	12-14d	0.11	0.37	5.47
300	0d-8d	0.47	1.56	5.12
300	8d-12d	0.06	0.199	20.10
300	12-14d	0.08	0.27	7.53
500	0d-8d	0.82	2.72	2.93
500	8d-12d	0.14	0.47	8.60
500	12-14d	0	0	∞
700	0d-8d	0.67	2.23	3.59
700	8d-12d	0.38	1.26	3.17
700	12-14d	0	0	∞
900	0d-8d	0.95	3.16	2.54
900	8d-12d	0.06	0.19	20.07
900	12-14d	0.18	0.59	3.35
1000	0d-8d	0.75	2.49	3.21
1000	8d-12d	0.12	0.39	10.03
1000	12-14d	0.1	0.33	6.02

Table 3.3 Doubling time (d) and No. of doublings of Oak (*Quercus robur* L.) embryogenic suspension cells in varying concentrations of kanamycin antibiotic in a 14d cell growth cycle.

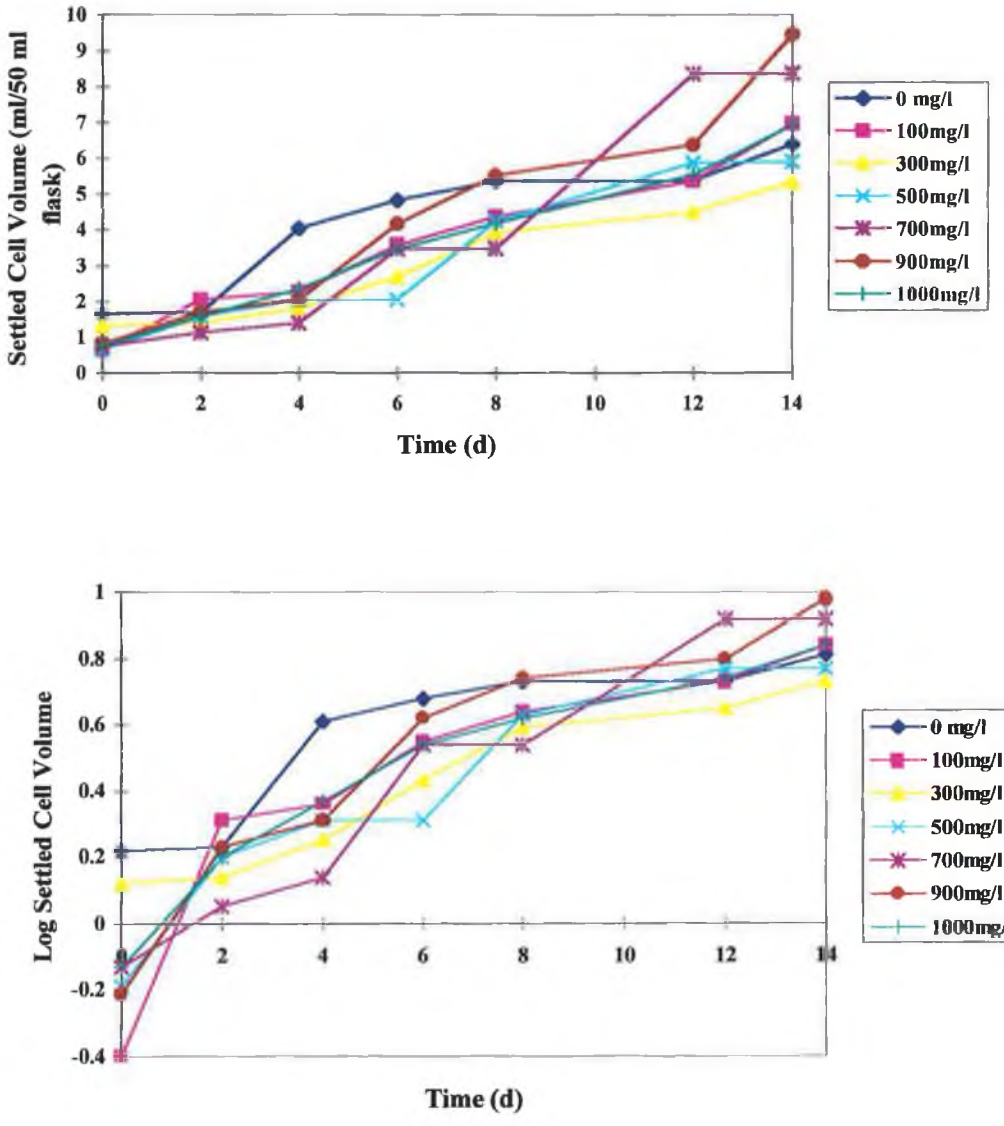
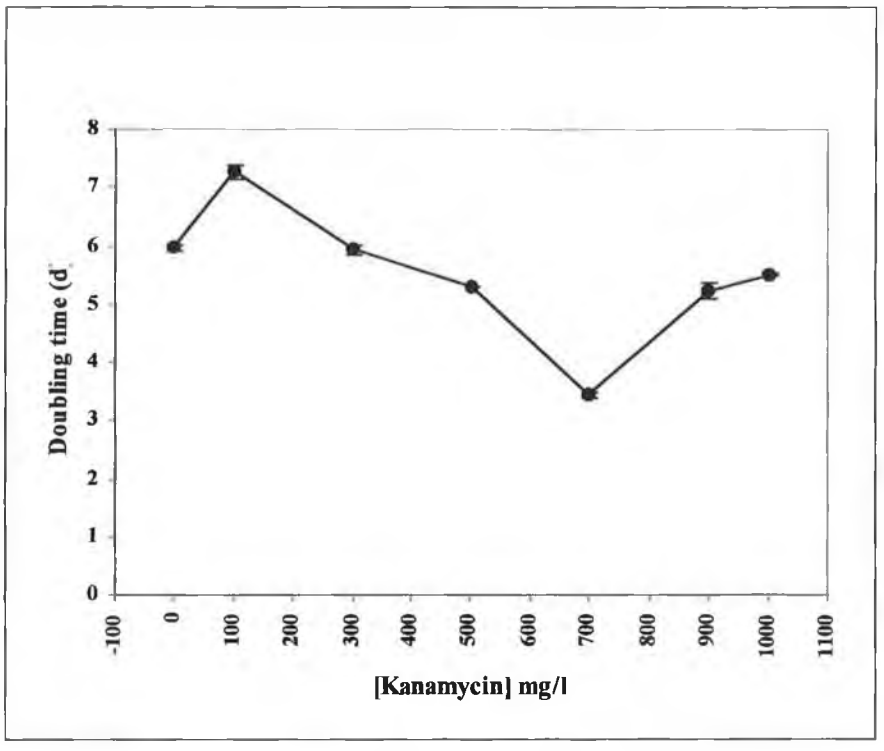


Figure 3.5

Growth of Oak (*Quercus robur* L.) embryogenic suspension cultures in different concentrations of kanamycin antibiotic over time. Results were recorded from the average of 5 flasks per kanamycin concentration (50 cm^{-3} medium per flask). Duplicate experiments did not differ significantly. Due to error bar overlapping, they were omitted from the graph. The units $mgdm^{-3}$ are consistent with mg/l.



DT

Tukey HSD[a]

KAN	N	Subset for alpha = .05			
		1	2	3	4
700.00	10	3.4474			
900.00	10		5.2330		
500.00	10		5.2986		
1000.00	10		5.5100		
300.00	10			5.9343	
.00	10			5.9768	
100.00	10				7.2692
Sig.		1.000	.204	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Figure 3.6

Mean doubling time (d) of Oak (*Quercus robur* L.) embryogenic suspension cultures from 4d-12d in varying concentrations of kanamycin antibiotic. Experiments were carried out in duplicate. Repeat experiments did not differ significantly and standard errors of the mean are included. Results of Tukey's HSD test are outlined in the statistical table, doubling times in the same column are significantly different from doubling times in other columns but not from each other.

3.2.5 Growth of Oak (*Quercus robur* L.) embryogenic suspension cultures in varying concentrations of paromomycin antibiotic

Since growth of Oak embryogenic suspension cells occurred in high concentrations of kanamycin antibiotic, the search for an antibiotic that was capable of selecting transformed Oak embryogenic cells was continued. Gonzalez *et al.*, 1998 successfully selected transformed Cassava plants on paromomycin containing medium. We suspected that paromomycin may be a useful selective antibiotic for selecting transformed Oak cultures.

Experiments were set up in exactly the same manner as the previous two experiments on growth of Oak embryogenic suspensor cells in media containing antibiotics. Settled cell volumes were measured every 2d for a 16d growth period. Results for the growth of Oak embryogenic suspension cultures in paromomycin antibiotic are presented below.

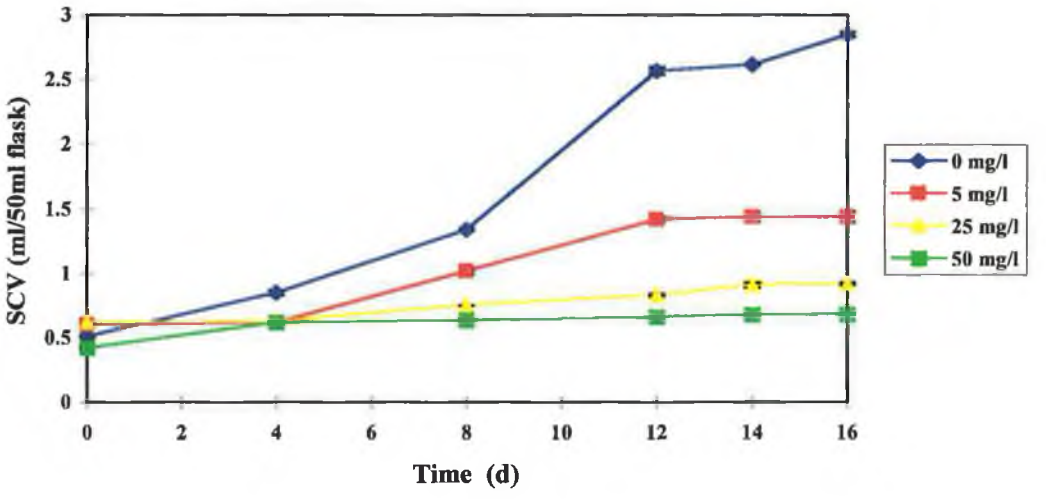
Figure 3.7 outlines the growth of Oak embryogenic suspensor cells in concentrations of paromomycin antibiotic varying from 0 $mgdm^{-3}$-50 $mgdm^{-3}$. Table 3.4 shows number of doublings and cell doubling times over the 16d growth cycle. ANOVA results showed that the effects of paromomycin concentration and time were both highly significant on the growth of Oak suspension cultures ($p<0.05$). The graph shows that for all concentrations cells grew from 0d-4d. However for 50 $mgdm^{-3}$ paromomycin concentration growth ceased after 4d and did not continue at any stage during the cycle. Growth of cells at 25 $mgdm^{-3}$ paromomycin continued slowly up to 12d and then levelled off completely. The effect of paromomycin concentration on the doubling time of cultures is outlined in Fig. 3.8. The

graph shows that there was a significant increase in doubling times from 0mgdm^{-3}-50mgdm^{-3} paromomycin. Tukey's HSD test shows that doubling times recorded at 0 mgdm^{-3} are significantly different to those recorded for 25 mgdm^{-3} and 50 mgdm^{-3} but not 5 mgdm^{-3} paromomycin.

Cessation of growth of Oak embryogenic suspension cells in concentrations of paromomycin as low as 25 mgdm^{-3}-50 mgdm^{-3} makes paromomycin a suitable antibiotic for the selection of transformed Oak embryogenic cells.

[Paromomycin] $mgdm^{-3}$	Time from start	Log increase in settled cell volume	No. SCV doublings in the time frame	Doubling Time (d)
0	0d-8d	0.42	1.39	5.73
0	8d-12d	0.28	0.93	4.30
0	12d-16d	0.04	0.13	30.30
5	0d-8d	0.23	0.76	10.47
5	8d-12d	0.14	0.47	8.60
5	12d-16d	0.01	0.03	120.48
25	0d-8d	0.08	0.27	30.11
25	8d-12d	0.05	0.17	24.08
25	12d-16d	0.04	0.13	30.12
50	0d-8d	0.19	0.63	12.67
50	8d-12d	0.01	0.03	120.48
50	12d-16d	0.01	0.03	120.48

Table 3.4 Doubling time (d) and No. of doublings of Oak (*Quercus robur* L.) embryogenic suspension cells in varying concentrations of paromomycin antibiotic in a 16d cell growth cycle.



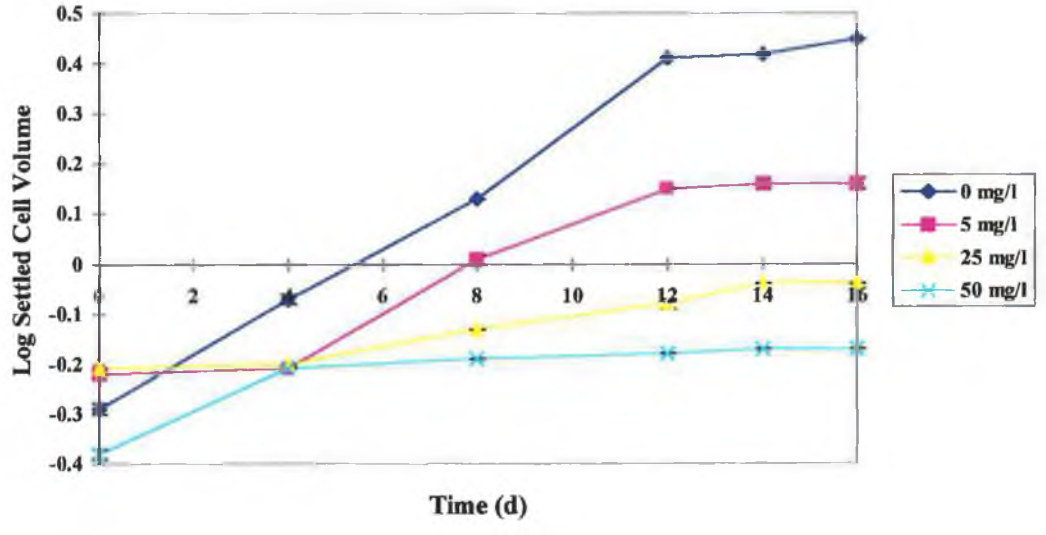
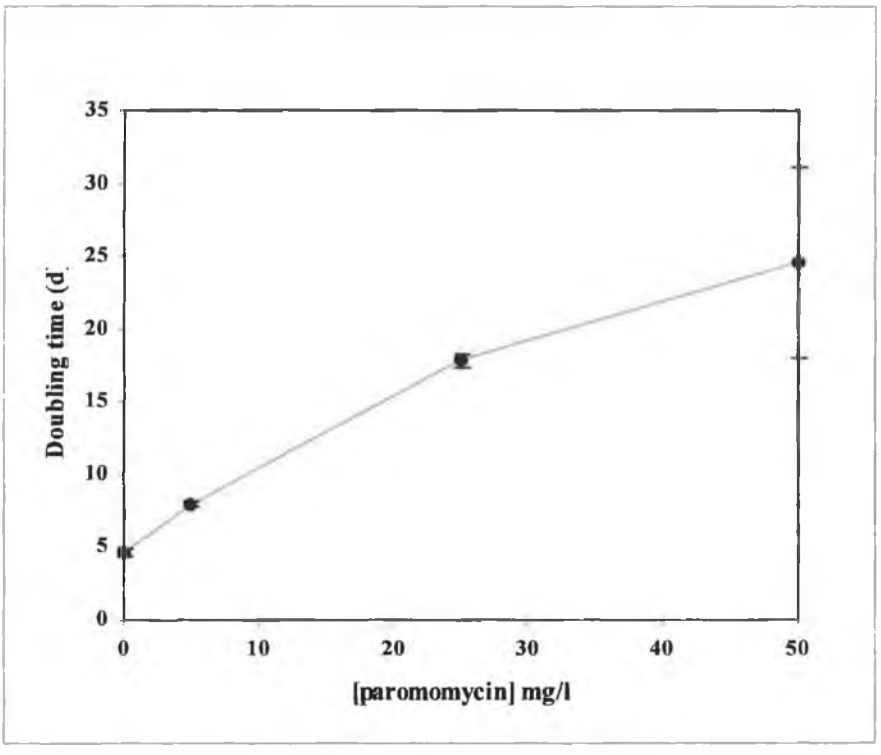


Figure 3.7
Growth of Oak (*Quercus robur* L.) embryogenic suspension cultures in different concentrations of paromomycin antibiotic over time. Results are the average of 5 flasks per paromomycin concentration (50 cm^{-3} medium per flask). The experiment was carried out in duplicate and standard error bars of the mean are included. The units $mgdm^{-3}$ are consistent with mg/l.



DT

Tukey HSD[a]

PAROMOMY	N	Subset for alpha = .05		
		1	2	3
.00	10	4.6407		
5.00	10	7.9883	7.9883	
25.00	10		17.8502	17.8502
50.00	10			24.6459
Sig.		.888	.165	.469

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Figure 3.8

Mean doubling times (d) of Oak (*Quercus robur* L.) embryogenic suspension cultures from 0d-12d in different concentrations of paromomycin antibiotic. Experiments were carried out in duplicate. Repeat experiments did not differ significantly and standard errors of the mean are included. Results of Tukey's HSD test are outlined in the statistical table, doubling times in the same column are significantly different from doubling times in other columns but not from each other.

3.2.6 Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses

Growth of Sitka Spruce embryogenic suspensor masses was investigated. The growth of ESMs in liquid culture has previously been reported (Krogstrup, 1988) however, growth can vary in differing environments and therefore it was necessary to evaluate growth in the environment in which all subsequent experiments would be carried out. Assessment of the growth characteristics of Sitka Spruce ESMs in liquid culture enables the estimation of optimum subculture times to maintain embryogenic potential in cultures and in the avoidance of unnecessary cell necrosis. A standard growth curve could be utilised in later experiments as a reference to cell growth in media in the absence of additional components.

From 7d old Sitka Spruce ESM liquid cultures 1:2, 1:4 and 1:8 dilutions were prepared in 250 cm^3 Erlenmeyer flasks. Settled cell volumes were recorded routinely for a 22d growth cycle. Flasks were cultured in the growth room.

Figure 3.9 outlines a 22d growth cycle of Sitka Spruce embryonal suspensor masses. The graphs indicated that the lower cell dilutions (1:2,1:4) started growing immediately and grew steadily right through the cell growth cycle. Higher cell dilutions (1:8) showed a lag phase from 0d-5d and then started to grow at a much faster rate than those cells at higher cell dilutions. Figure 3.10 shows initial settled cell volume plotted against doubling times. Results show that the greater the initial settled cell volume the longer the cell doubling time. These results correlate with results outlined in Figure 3.9.

Experiments analysing the effects of antibiotics on the growth of Sitka Spruce embryonal suspensor masses could now be examined.

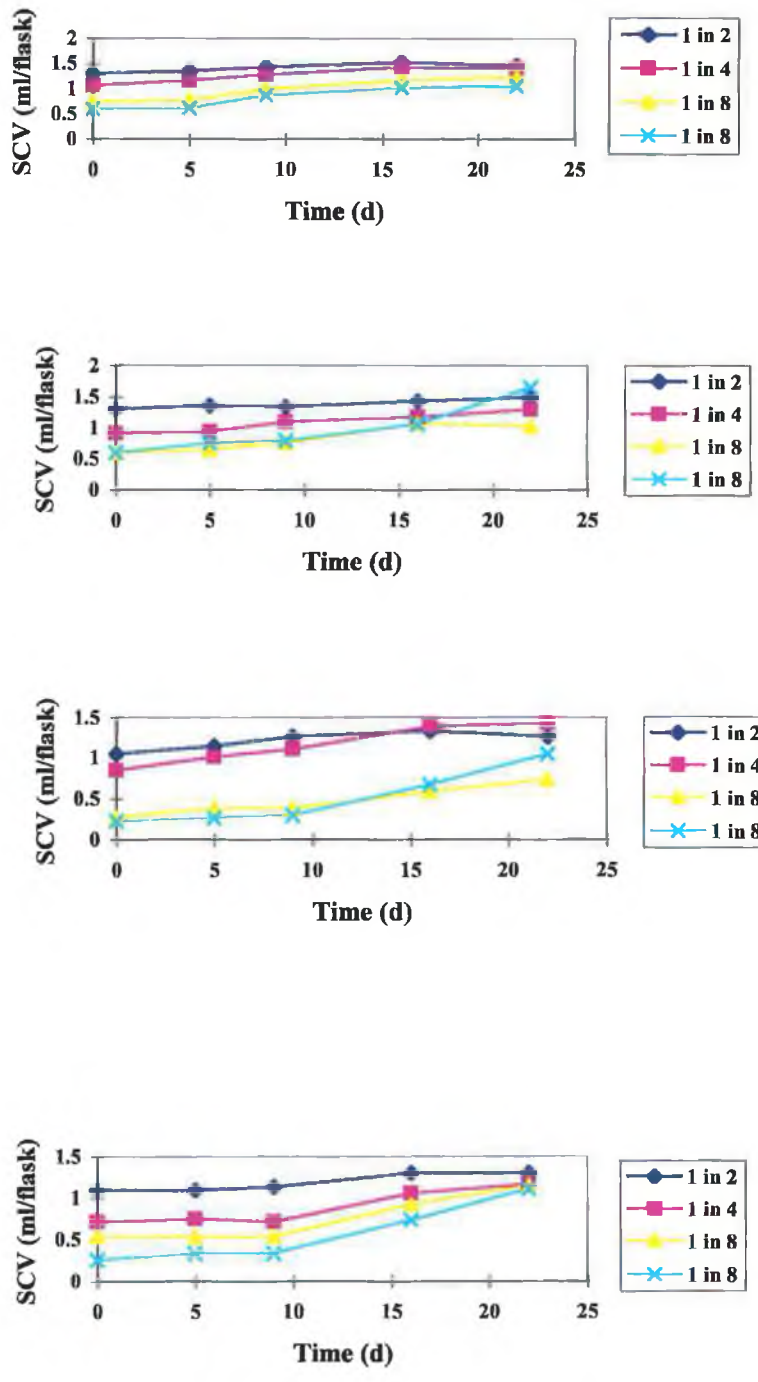


Figure 3.9

Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in varying dilutions over time following innoculation at different cell dilutions.

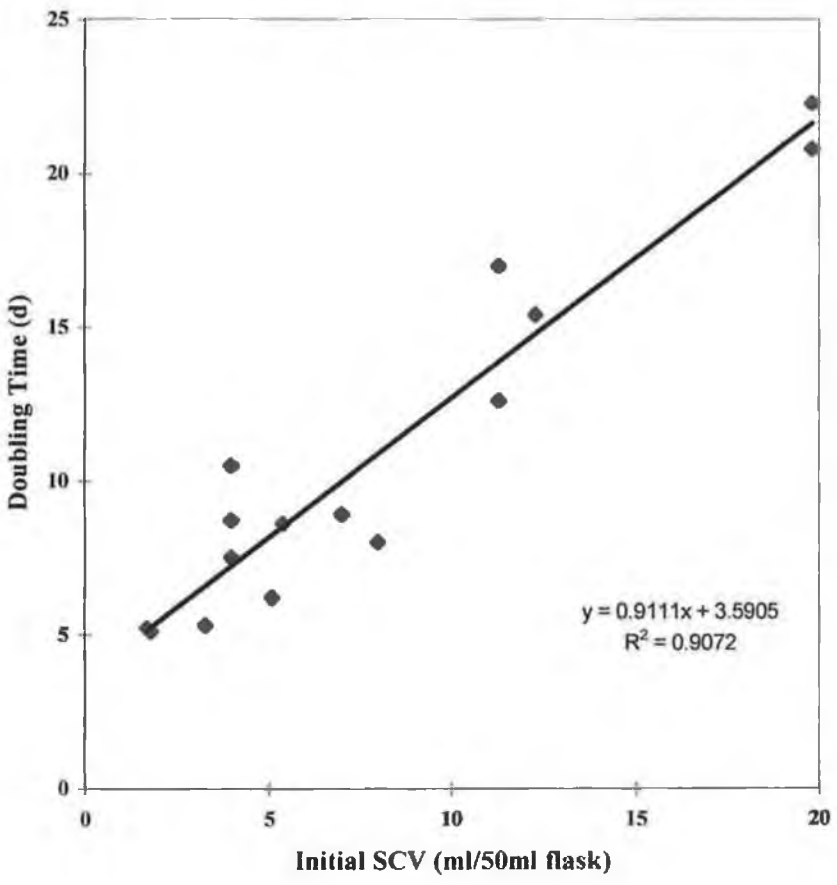


Figure 3.10

Graph showing initial settled cell volume of flasks (50 cm^{-3} medium) of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass against subsequent doubling times.

3.2.7 Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in varying concentrations of cefotaxime antibiotic

Again as with Oak embryogenic suspension cultures the growth of Sitka Spruce embryogenic suspensor cells in cefotaxime antibiotic needed to be assessed.

Sitka Spruce embryogenesis maintenance medium was prepared as normal. Cefotaxime concentrations ranging from 0 $mgdm^{-3}$ -1000 $mgdm^{-3}$ were filter sterilised into autoclaved medium. Conditioned medium was removed from 10d old Sitka Spruce suspension cultures and subculturing was carried out using the new embryogenesis medium with varying cefotaxime concentrations. Flasks were removed every 2d from the culture room and settled cell volumes measured for a 14d growth cycle. The experiment was carried out in duplicate. Results are presented below.

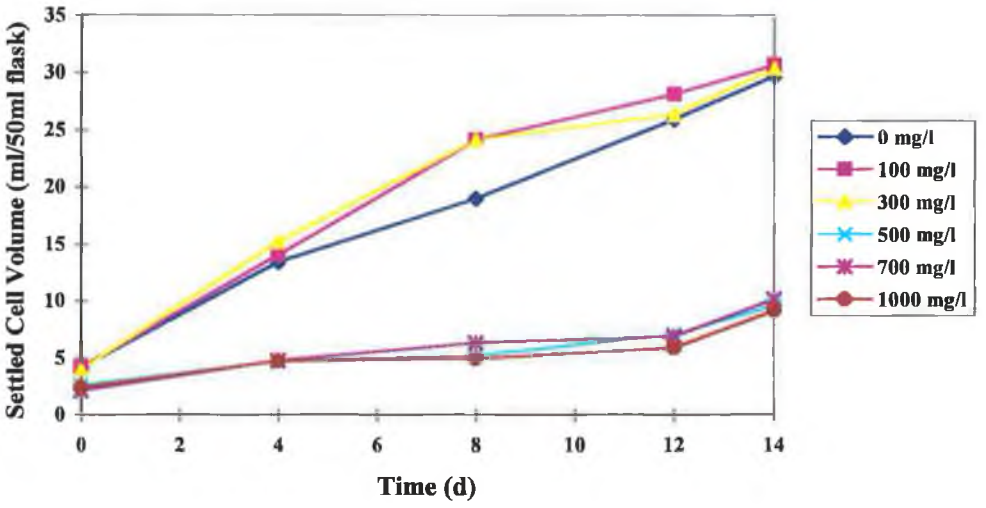
Figure 3.11 shows the growth of Sitka Spruce embryonal suspensor masses in concentrations of cefotaxime ranging from 0 $mgdm^{-3}$–1000 $mgdm^{-3}$. Initial settled cell volumes varied from 2.066 cm^3–4.15 cm^3 per 50 cm^{-3}. The graph shows that growth of embryonal suspensor masses was not inhibited by cefotaxime concentrations up to 1000 $mgdm^{-3}$ in a 14d growth cycle. However ANOVA results showed that the effects of cefotaxime concentration and time were both highly significant ($p<0.05$). Table 3.5 outlines the cell doubling times and number of cell doublings in the 14d growth cycle. The graph shows that for 0 $mgdm^{-3}$, 100 $mgdm^{-3}$, 300 $mgdm^{-3}$ cefotaxime treatments, the initial SCV was higher than that of 500 $mgdm^{-3}$, 700 $mgdm^{-3}$ and 1000 $mgdm^{-3}$ cefotaxime. Doubling times showed that the higher concentrations of cefotaxime suffered a lag phase from 8d-12d in the growth cycle due to this lower initial SCV but experienced a much higher growth rate towards the end of the growth cycle unlike the lower cefotaxime concentrations. Figure

3.12 shows the effect of cefotaxime concentration on the doubling time of cell growth. Although ANOVA results show that the effect of cefotaxime concentration is highly significant, the graph shows that it is not having a large effect on cell doubling time with increasing concentration. Tukey's HSD test shows that the slowest doubling time was recorded for 100 $mgdm^{-3}$ cefotaxime but doubling times for all other concentrations with the exception of 0 $mgdm^{-3}$ and 100 $mgdm^{-3}$ were not significantly lower.

The continued growth of Sitka Spruce ESMs in concentrations of cefotaxime as high as 1000 $mgdm^{-3}$ allows for its use as an *Agrobacterium* inhibitor following *Agrobacterium*-mediated transformation. The choice of an antibiotic for selection of transformed Sitka Spruce ESMs is investigated in the set of experiments.

[Cefotaxime] $mgdm^{-3}$	Time from start	Log increase in settled cell volume	No. SCV doublings in the time frame	Doubling Time (d)
0	0d-8d	0.66	2.19	3.65
0	8d-12d	0.13	0.43	9.26
0	12d-14d	0.06	0.19	10.03
100	0d-8d	0.76	2.52	3.17
100	8d-12d	0.07	0.23	17.20
100	12d-14d	0.04	0.13	15.05
300	0d-8d	0.78	2.59	3.08
300	8d-12d	0.04	0.13	30.12
300	12d-14d	0.06	0.19	10.03
500	0d-8d	0.31	1.03	7.77
500	8d-12d	0.13	0.43	9.26
500	12d-14d	0.13	0.43	4.63
700	0d-8d	0.48	1.59	5.01
700	8d-12d	0.04	0.13	30.30
700	12d-14d	0.17	0.56	30.54
1000	0d-8d	0.32	1.06	7.52
1000	8d-12d	0.08	0.26	15.05
1000	12d-14d	0.19	0.63	3.16

Table 3.5 Doubling time (d) and No. of doublings of Sitka Spruce (*Picea sitchensis*) embryonal suspensor masses in varying concentrations of cefotaxime antibiotic in a 14d cell growth cycle



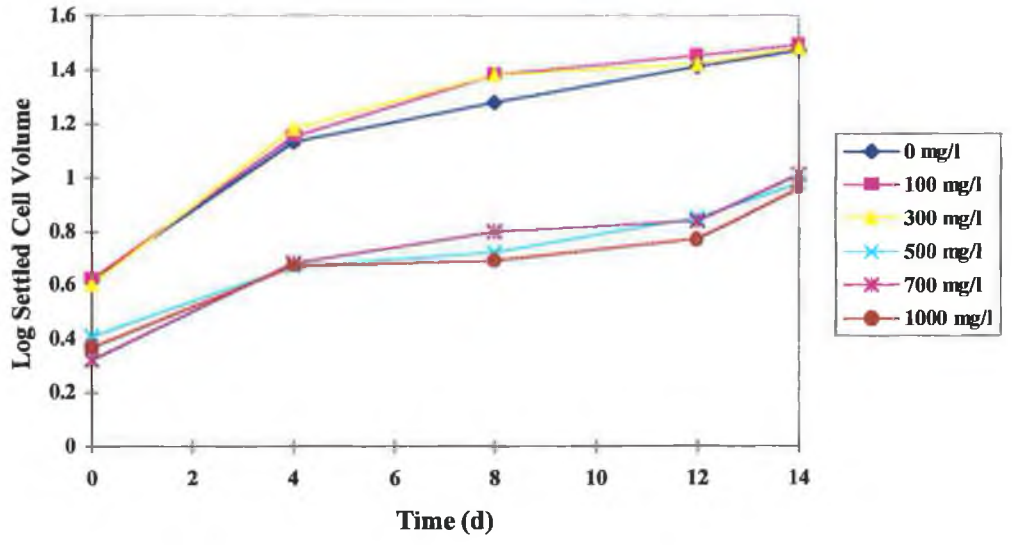
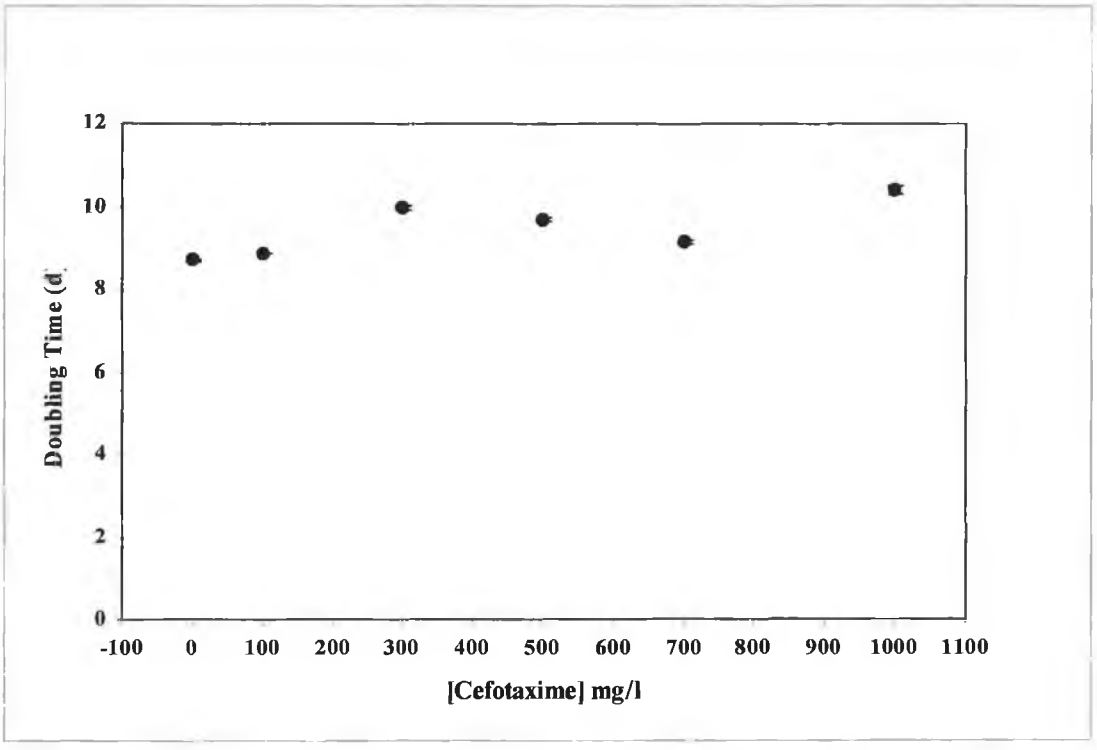


Figure 3.11

Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass in different concentrations of cefotaxime antibiotic. Results were recorded from the average of 5 flasks (50 cm^{-3} medium per flask) per cefotaxime concentration. Duplicate experiments did not differ significantly in growth and due to error bar overlapping, they were omitted from the graph. The units $mgdm^{-3}$ are consistent with mg/l.



DT

Tukey HSD[a]

CEF	N	Subset for alpha = .05				
		1	2	3	4	5
.00	10	8.7101				
100.00	10	8.8651				
700.00	10		9.1381			
500.00	10			9.6679		
300.00	10				9.9746	
1000.00	10					10.4095
Sig.		.385	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Figure 3.12

Mean doubling time (d) of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass in varying concentrations of cefotaxime antibiotic. Experiments were carried out in duplicate. Standard errors of the mean are included. Results of Tukey's HSD test are outlined in the statistical table, doubling times in the same column are significantly different from doubling times in other columns but not from each other.

3.2.8 Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in varying concentrations of kanamycin antibiotic

Agrobacterium-mediated transformation of Sitka Spruce embryonal suspensor masses was carried out with the LBA4404::pBI121 strain therefore growth of Sitka cells in the presence of kanamycin needed to be assessed.

Kanamycin concentrations ranging from 0 $mgdm^{-3}$-1000 $mgdm^{-3}$ were filter sterilised into autoclaved Sitka Spruce embryo maintenance medium. Flasks were prepared as with the cefotaxime experiment above and again 5 flasks were sampled for each kanamycin concentration. Flasks were removed every 2d from the culture room and settled cell volumes recorded for a total growth period of 14d. The experiment was carried out in duplicate. Results for growth of Sitka Spruce ESM suspension cultures in varying concentrations of kanamycin antibiotic are presented below.

Figure 3.13 shows the growth of Sitka Spruce embryonal suspensor mass in concentrations of kanamycin antibiotic varying from 0 $mgdm^{-3}$–1000 $mgdm^{-3}$. The graph indicates the continued growth of suspensor mass in all concentrations of kanamycin in a 14d growth cycle. ANOVA results indicated that kanamycin concentration and time had a highly significant effect on cell growth ($p<0.05$). Initial settled cell volumes varied from 2.254 cm^3–4.1 cm^3. Table 3.6 outlines the cell doubling times and number of cell doublings for each antibiotic concentration. The graph (Fig 3.13) shows a lag in growth for all kanamycin concentrations from 0d-2d followed by a steady increase to 14d. Figure 3.14 shows the effect of kanamycin concentration on the doubling time of Sitka Spruce cultures. Doubling times for all kanamycin concentrations with the exception of 700 $mgdm^{-3}$ are lower than for 0 $mgdm^{-3}$. This indicates as with Oak embryogenic cultures that rather than

having a detrimental effect on cell growth appears to be benefiting cell growth. Tukey's HSD test shows that Sitka Spruce embryonal suspensor mass are growing at their fastest rate in the presence of 900 $mgdm^{-3}$ kanamycin.

Kanamycin did not hinder growth of Sitka Spruce ESMs to a high enough extent to allow its use for the selection of transformed ESMs. The next experiment examines growth in paromomycin antibiotic to investigate its potential as a selective antibiotic for Oak and Sitka Spruce transformed embryogenic suspension cultures.

[Kanamycin] $mgdm^{-3}$	Time from start	Log increase in settled cell volume	No. SCV doublings in the time frame	Doubling Time (d)
0	0d-8d	0.79	2.62	3.05
0	8d-12d	0.13	0.43	9.26
0	12d-14d	0.06	0.19	10.03
100	0d-8d	0.71	2.36	3.39
100	8d-12d	0.11	0.37	10.94
100	12d-14d	0.07	0.23	8.60
300	0d-8d	0.73	2.43	3.29
300	8d-12d	0.15	0.49	8.03
300	12d-14d	0.04	0.13	15.06
500	0d-8d	0.74	2.46	3.26
500	8d-12d	0.13	0.43	9.26
500	12d-14d	0.04	0.13	15.06
700	0d-8d	0.62	2.06	3.88
700	8d-12d	0.12	0.39	10.03
700	12d-14d	0.04	0.13	15.05
900	0d-8d	0.86	2.85	2.80
900	8d-12d	0.14	0.47	8.60
900	12d-14d	0.03	0.09	20.07
1000	0d-8d	0.83	2.75	2.91
1000	8d-12d	0.1	0.33	12.04
1000	12d-14d	0.04	0.13	15.06

Table 3.6 Doubling time (d) and No. of doublings of Sitka Spruce (*Picea sitchensis*) embryonal suspensor masses in varying concentrations of kanamycin antibiotic in a 14d cell growth cycle

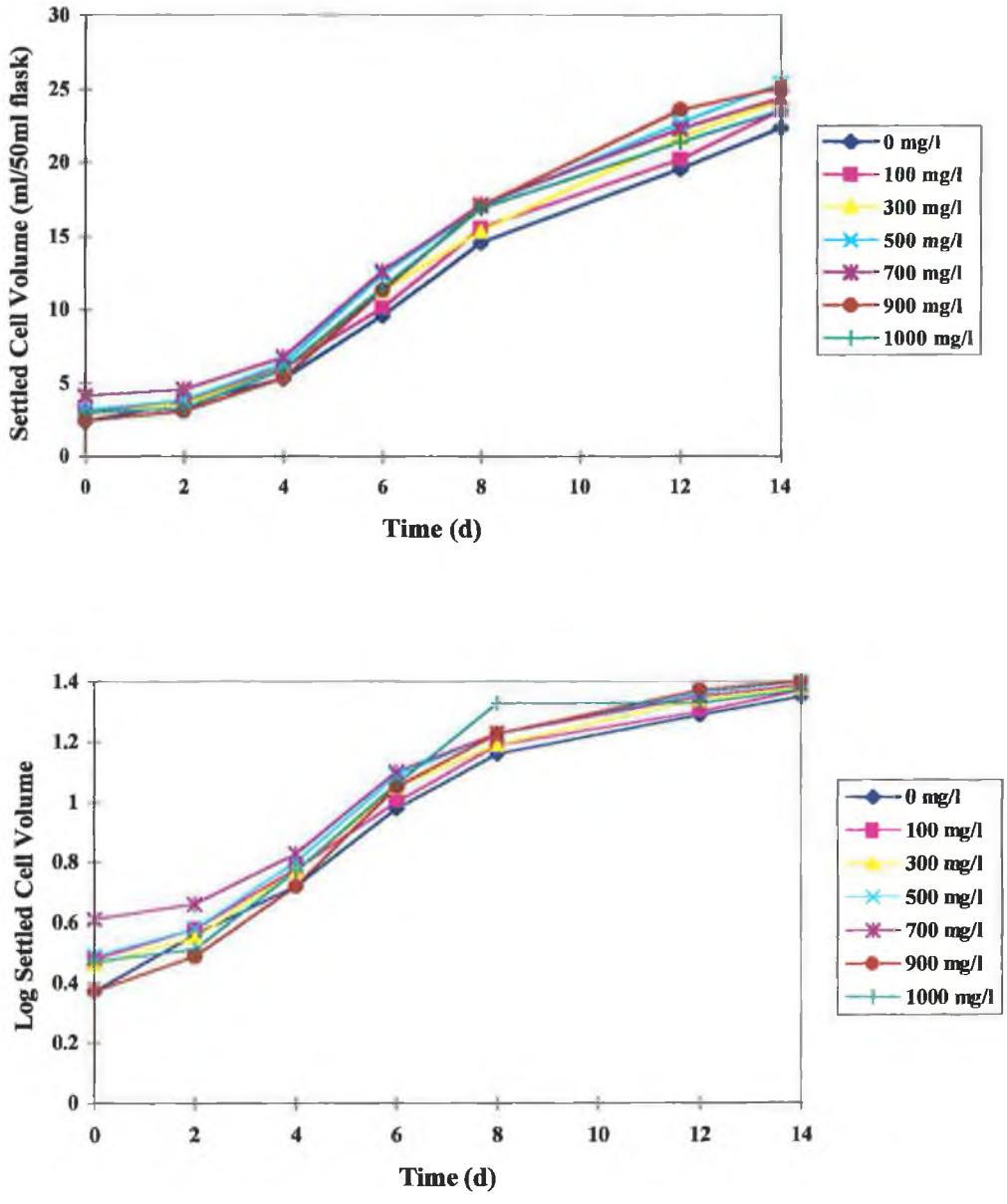
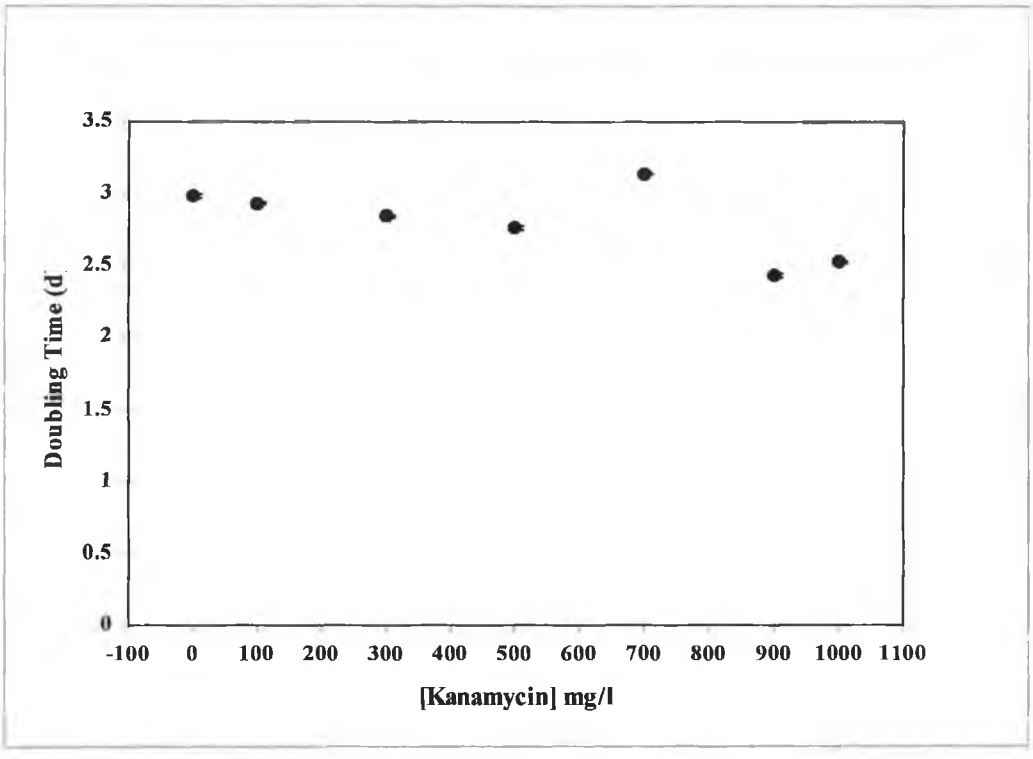


Figure 3.13

Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass in different concentrations of kanamycin antibiotic. Results were recorded from the average of 5 flasks (50 cm^{-3} medium) per kanamycin concentration. Duplicate experiments did not differ significantly and error bars were omitted due to overlapping. The units $mgdm^{-3}$ are consistent with mg/l.



DT

Tukey HSD[a]

KAN	N	Subset for alpha = .05						
		1	2	3	4	5	6	7
900.00	10	2.4297						
1000.00	10		2.5269					
500.00	10			2.7678				
300.00	10				2.8442			
100.00	10					2.9382		
.00	10						2.9886	
700.00	10							3.1382
Sig.		1.000	1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Figure 3.14

Mean doubling time (d) of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass in different concentrations of kanamycin antibiotic. Experiments were carried out in duplicate. Standard errors of the mean are included. Results of Tukey's HSD test are outlined in the statistical table, doubling times in the same column are significantly different from doubling times in other columns but not from each other.

3.2.9 Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in varying concentrations of paromomycin antibiotic

Results outlined in the previous section revealed that kanamycin is not sufficiently selective for transformed Sitka Spruce ESMs. The effect of paromomycin, another aminoglycoside antibiotic on the growth of sitka ESMs is investigated in the following experiment.

Experiments were set up as with the previous two experiments on cell growth in the presence of antibiotics cefotaxime and kanamycin. Settled cell volumes were recorded every 2d for a 16d growth period. The experiment was repeated following the completion of the first experiment. Results on the growth of Sitka Spruce embryogenic suspensor masses in different concentrations of paromomycin antibiotic are presented below.

Figure 3.15 outlines the growth of Sitka Spruce embryonal suspensor mass in concentrations of paromomycin between 0 $mgdm^{-3}$–50 $mgdm^{-3}$. The graph shows continued growth of embryonal masses to 14d in the absence of paromomycin. ANOVA results showed that the effects of paromomycin concentration and time were both highly significant on the growth of Sitka Spruce ESMs (<0.05). Table 3.7 outlines the number of doublings of settled cell volume and doubling times. Embryogenic suspensor mass in all concentrations of paromomycin grew from 0d-4d with doubling times varying from 3d (0 $mgdm^{-3}$)-20d (0.3 $mgdm^{-3}$). All concentrations above 1 $mgdm^{-3}$ ceased to continue growth after 8d whereas 0 $mgdm^{-3}$ continued growing. ESMs in 0.3 $mgdm^{-3}$ and 1 $mgdm^{-3}$ stopped growing at 12d.

Inhibition of the growth of Sitka Spruce embryogenic suspensor cells in concentrations of paromomycin as low as 3 $mgdm^{-3}$ after 8d suggests that paromomycin may be a suitable antibiotic for selection of ESM transformed cells.

Results in this chapter have identified antibiotics for the suppression of *Agrobacterium* growth and for the selection of transformed cells thereby facilitating experiments into *Agrobacterium*-mediated transformation. The following chapter outlines a number of transformation experiments on Sitka Spruce embryonal suspensor masses.

[Paromomycin] $mgdm^{-3}$	Time from start	Log increase in settled cell volume	No. SCV doublings in the time frame	Doubling Time (d)
0	0d-4d	0.34	1.13	3.10
0	4d-8d	0.29	0.96	4.15
0.3	0d-4d	0.06	0.19	20.14
0.3	4d-8d	0.09	0.29	13.38
1	0d-4d	0.09	0.29	13.42
1	4d-8d	0.13	0.43	9.28
3	0d-4d	0.15	0.49	8.03
3	4d-8d	0.05	0.17	24.09
5	0d-4d	0.48	1.59	2.51
5	4d-8d	0	0	∞
25	0d-4d	0.27	0.89	4.46
25	4d-8d	0.09	0.19	20.07
50	0d-4d	0.07	0.23	17.20
50	4d-8d	0	0	∞

Table 3.7 Doubling time (d) and No. of doublings of Sitka Spruce (*Picea sitchensis*) embryonal suspensor masses in varying concentrations of paromomycin antibiotic in a 14d cell growth cycle

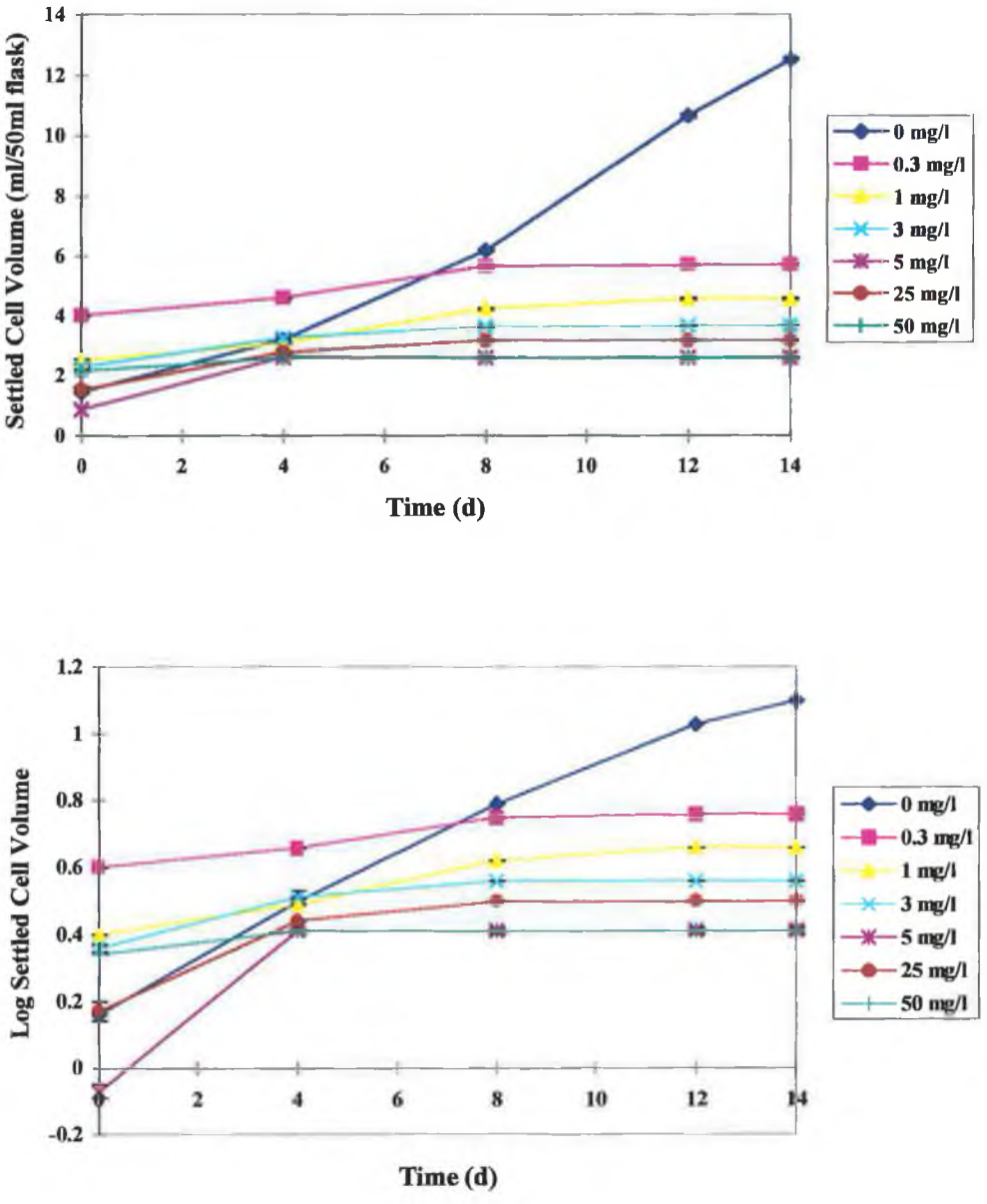


Figure 3.15

Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass in varying concentrations of paromomycin antibiotic. Results were recorded from the average of 5 flasks (50 cm^{-3} medium per flask) per paromomycin concentration. Experiments were carried out in duplicate and standard errors of the mean were included. The units $mgdm^{-3}$ are consistent with mg/l.

3.3 Discussion

Growth of Oak and Sitka Spruce embryogenic cells in liquid culture reduces the variation caused by gradients of chemical and physical factors and accelerates experimental response time as compared with solid medium culture which will ensure a more reliable experimental model system. Paromomycin interacts with Gelrite, the gelling agent routinely used for our solid cultures and is precipitated (Keith Fuell, per. comm.). Somatic embryos of many conifer species can be grown in liquid suspension culture. In this state cultures grow more rapidly than in solid phase medium (Lulsdorf *et al.*, 1992).

The effect of cefotaxime on the suppression of *Agrobacterium* growth in culture medium was observed. All concentrations higher that 0 $mgdm^{-3}$ proved detrimental to *Agrobacterium* growth. Kokro *et al.*, 1998 also found that concentrations of cefotaxime between 25-50 $mgdm^{-3}$ inhibited the growth of *Agrobacterium* in the dark. Higher concentrations were however used in our transformation experiments (500 $mgdm^{-3}$) as cefotaxime is degraded by the light.

Oak embryogenic suspension cultures show an increase in growth from 0d to 12d in a 14d growth cycle. The highest growth rate was recorded between 0d-4d with a doubling time of 2.23d. Growth levelled off between 12d-14d and these results suggest that cells should be subcultured every 10d to maintain a consistent growth in all cultures. Chalupa V, 1990 cultured embryos of *Quercus robur*, L on MS solid medium supplemented with 0.5 $mgdm^{-3}$, 1.0 $mgdm^{-3}$ and 2.0 $mgdm^{-3}$ benzylaminopurine. Chalupa's embryogenic callus cultures were transferred every 4–6 weeks onto fresh medium.

Growth cycles of Sitka Spruce embryonal suspensor masses show an increase in

settled cell volume from 0d–22d. Results show that more dilute cell suspension cultures have a lag phase from 0d-5d but then grow more rapidly compared to less dilute cultures which grow at a steady rate throughout the cycle. Although Sitka Spruce embryonal suspensor masses were still growing at 22d subculturing was carried out every 10d as browning of cultures occurred if they were left for too long between subcultures. Krogstrup *et al.*, 1988 maintained and proliferated Sitka Spruce ESMs on BMI–S1 liquid medium with the cultures being sub-divided and subcultured at 7d to 10d intervals. Krogstrup also found that the growth rate was dependent on age, starting inoculum and size. This however was not found for Oak embryogenic suspensions (Fig 3.2).

Growth of Oak and Sitka Spruce suspension cultures was quantified as outlined in section 2.3.1 using a settled cell volume method. In his quantification of the growth of embryogenic cell suspensions of *Picea sitchensis* consisting primarily of stage 1 proembryos Krogstrup *et al.*, 1988 also recorded settled cell volume but in a different manner. Krogstrup measured settled cell volume by pouring 100 cm^3 of culture into a sterile 100 cm^3 measuring cylinder and allowing it to sediment for 30min. The resulting sedimented settled cell volume was used as a non-destructive quantitative measurement of cell suspension growth. The method of measuring settled cell volume utilised in our work was a less invasive method of quantifying growth as it did not require removal of cultures from their flasks (Gilissen *et al.*, 1983).

The growth of Oak embryogenic suspension cultures and Sitka Spruce embryonal suspensor masses in different concentrations of cefotaxime antibiotic was measured with the aim of using cefotaxime as an *Agrobacterium* inhibitor following transformation. *Agrobacterium* must be eliminated as bacteria will contaminate the culture, continuously transform cells, and are a potential environmental threat if released into the environment.

When considering the use of *Agrobacterium* as a tool in genetic engineering, it is now imperative to also address the magnitude of risk posed to the environment in releasing transgenic plants. If all of the bacteria are not eliminated after transformation then the release of these plants may also result in the release of the *Agrobacterium* (Barrett *et al.*, 1997). Results showed that in the case of both Oak and Sitka Spruce suspension cultures cefotaxime did not affect their growth up to levels as high as 1000 $mgdm^{-3}$. However ANOVA results suggested that cefotaxime had a highly significant effect on cell growth. Results on the effects of antibiotic concentration on cell doubling time showed that doubling times for Oak cells for all concentrations with the exception of 700 $mgdm^{-3}$ only varied between 3.02 d (500 $mgdm^{-3}$) and 3.73 d (100 $mgdm^{-3}$). The difference in doubling times for Sitka Spruce ESMs from 0 $mgdm^{-3}$-1000 $mgdm^{-3}$ was recorded at 1.69 d. Cefotaxime is a β-lactam which is considered to be non-toxic to plant cells due to it's specific action on bacterial cell walls. Sarma *et al.*, 1995 in their studies to analyse the effects of cefotaxime and carbenicillin on somatic embryogenesis of Sitka Spruce, found that at levels of 500 $mgdm^{-3}$ cefotaxime, no significant differences were observed between cefotaxime and the control when tissue growth was measured however effects ondeveloping somatic embryos was recorded.. Roest & Evers, 1991 reported the use of 300 $mgdm^{-3}$ cefotaxime for the elimination of *Agrobacterium* in *Agrobacterium*-mediated transformation of *Quercus robur* L. nodal stem explants.

The ideal antibiotic for inhibiting *A. tumefaciens* in genetic transformation should be highly effective in suppression of bacterial growth, have a negligible effect on the plant growth and regeneration and be chemically stable in culture (Cheng *et al.*, 1998). Cefotaxime fits all of these criteria. Oak and Sitka Spruce cultures need to be subcultured on antibiotic containing medium at least five to six times to ensure bacterial suppression.

The high cost of the cefotaxime antibiotic makes this genetic transformation method very costly. Studies on the use of timentin as an alternative antibiotic for the inhibition of *Agrobacterium* (Nauerby *et al.*, 1997; Cheng *et al.*, 1998) in Tobacco plants show that it can inhibit *Agrobacterium* growth at level as low as 150 $mgdm^{-3}$ or can work in combination with cefotaxime in ratios of 2:1, with the concentration of cefotaxime being half of its normal concentration required. The affect of timentin on the growth of Oak or Sitka Spruce embryogenic suspension cultures is unknown however and studies would have to be carried out before it could be considered as a cheaper alternative to cefotaxime.

Agrobacterium-mediated transformation with the LBA4404::pBI121 strain results in the integration of a plasmid containing the *npt* II gene into the plant genome. This *npt* II gene confers resistance to a number of aminoglycoside antibiotics including kanamycin and paromomycin. In order to select transformed cells on medium containing either of these antibiotics it is important that untransformed cells do not grow in their presence. Results show that in the presence of kanamycin at concentrations as high as 1000 $mgdm^{-3}$ growth of both Oak embryogenic suspension cultures and Sitka Spruce embryonal suspensor masses was unaffected. For both Oak and Sitka Spruce embryogenic suspension cultures it appeared that kanamycin had a beneficial effect on cell growth. In contrast, in the presence of paromomycin antibiotic, growth of Oak embryogenic suspension cultures was inhibited at concentrations of 25 $mgdm^{-3}$ at 12d in a 16d growth cycle. Paromomycin also inhibited the growth of Sitka Spruce embryonal suspensor masses at concentrations as low as 3 $mgdm^{-3}$. Drake *et al.*, 1997 reported that Sitka Spruce ESMs transformed with *Agrobacterium* carrying a vector with the *npt* II gene survived exposure to kanamycin in the culture medium at 15 $mgdm^{-3}$ while non transformed ESMs failed to survive culture on medium with 5 $mgdm^{-3}$ of this antibiotic. Gonzalez *et al.*, 1998, selected transgenic Cassava plants on

medium containing 15.39 $mgdm^{-3}$ paromomycin following transformation with *Agrobacterium* strain ABI containing the binary vector pMON977 with the *npt* II gene as selectable marker.

These results indicate that cefotaxime at concentrations as high as 1000 $mgdm^{-3}$ can be used in the elimination of *Agrobacterium* strain LBA4404::pBI121 from Oak and Sitka Spruce embryogenic cultures in liquid medium without having any affect on their continued growth. The effects of this antibiotic on regeneration of embryogenic cultures will be discussed later. The continued growth of untransformed Oak and Sitka Spruce in liquid culture in the presence of kanamycin rules it out for use as a selectable agent following transformation. However, inhibition of growth of both Oak and Sitka Spruce embryogenic cultures in liquid medium in the presence of low levels of paromomycin should enable its use as a selectable agent following *Agrobacterium*-mediated transformation with the strain LBA4404::pBI121. It must be noted that stably transformed cultures of Oak and Sitka Spruce expressing NPT II are not yet available. Therefore it was not possible to include positive controls in the experiments on the effects of antibiotics on the growth of embryogenic suspension cultures. Transformed cultures will contain the NPT II gene which when expressed will allow for the continued growth of transformed cultures in medium containing paromomycinantibiotic.

3.4 Conclusions

In conclusion, the results described in this section quantify the growth of Oak and Sitka Spruce embryogenic cultures in liquid medium using a less destructive method than previously reported. Ideal subculturing times of 10d for Oak and Sitka Spruce embryonal suspension liquid cultures were estimated. Cefotaxime was identified as a suitable antibiotic for the inhibition of *Agrobacterium* growth in cultures following transformation. Continued growth of Oak and Sitka Spruce cultures in high levels of cefotaxime meant that as it did not affect growth it could be used for *Agrobacterium* inhibition. A concentration of 500 $mgdm^{-3}$ was decided on, as it was sufficiently high to kill the *Agrobacterium*. Paromomycin was identified as an antibiotic which inhibited the growth of non transformed Oak and Sitka Spruce embryogenic suspension cultures at low concentrations in the medium. Concentrations of 3 $mgdm^{-3}$ for Sitka Spruce and 30 $mgdm^{-3}$ for Oak were found sufficiently high to inhibit the growth of non-transformed cultures.

These results suggested that transformants of Oak and Sitka Spruce could be reliably selected using paromomycin and that the residual *Agrobacterium* could be removed using cefotaxime antibiotic.

Chapter 4

Agrobacterium-mediated Transformation of Oak (*Quercus robur* L.) and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Embryogenic Suspension Cultures.

4.1 Introduction

In 1983 the era of plant genetic engineering was initiated when *Agrobacterium*-mediated gene delivery was used for producing transgenic plants (Fraley *et al.*, 1983). Forest trees are crops for which genetic engineering offers great potential benefit (Huang *et al.*, 1991). Genetic engineering techniques have the potential to supplement traditional tree breeding programmes which are hindered by the long generation time, sexual incompatibilities and the large area needed for tree breeding.

The two major approaches to gene transfer are based either on the bacterial plant pathogen *Agrobacterium* (Chilton, 1983; Bevan, 1984) or various physical methods for direct introduction of DNA (Paszkowski and Saul, 1986; Klein *et al.*, 1987). Those gene transfer systems mediated by *Agrobacterium* are the most effective, the simplest to apply and the best understood (Shin *et al.*, 1994). *Agrobacterium* infection has been demonstrated in many conifers (Sederoff *et al.*, 1986; Loopstra *et al.*, 1990; Stomp *et al.*, 1990; Bergmann and Stomp, 1992; Tzfira *et al.*, 1996) but transgenic plants have been regenerated only for *Larix decidua* Mill. (Huang *et al.*, 1991). Evers *et al.*, 1988 reported some shoot formation after infection of stem explants of Oak with a wild strain of *A. rhizogenes*. Roest *et al.*, 1991 reported successful expression of *GUS* activity at the base of Oak (*Quercus robur* L.) explants 30d after infection with an *Agrobacterium tumefaciens* strain.

Direct gene transfer avoids the need for introduction of cloned DNA into the T-DNA of *Agrobacterium tumefaciens* Ti plasmid or its equivalent before insertion into the plant. Many reports have detailed transient expression by direct gene transfer for a number of conifer species (Bekkaoui *et al.*, 1990; Charest *et al.*, 1993; Duchesne and Charest,

1991,1992; Gupta *et al.*, 1988; Loopstra *et al.*, 1992; Stomp *et al.*, 1991; Wilson *et al.*, 1989).

Successful transformation requires the optimization of several factors that include *in vitro* culture systems, method of gene transfer and the expression of introduced genes in the plant tissue (Christov, 1992). Embryogenesis as a means of rapid micropropagation has been developed for many species and therefore embryogenic cultures are attractive targets for generating genetically transformed plants as embryogenic cultures are most amenable to somatic embryogenis. The *GUS* gene coding for β-glucuronidase (Jefferson, 1987) and the *npt* II gene coding for kanamycin resistance are the most frequently used genes for expression and selection in transformed plants.

Attempts to obtain transformed plants often fail because one of the transformation steps fails or the transformed genes are not expressed in the plant cell. Primach-Zachwiega *et al.*, 1991 reported that acetosyringone, vanillin, catechol, P-hydroxybenzoate, β-resocylate and proto-catachuate were all effective in enhancing the expression of several *vir* genes. Acetosyringone is a phenolic inducer of virulence (*vir*) gene expression in *Agrobacterium* and it has been demonstrated to promote transformation of dicotyledons (Sheikholeslam and Weeks, 1987; Drake *et al.*, 1997).

Genetic engineering methods can therefore augment conventional breeding of forest trees which proceeds at a rather slow pace for the growing demand on this diminishing resource. This chapter examines the possibilities of *Agrobacterium*-mediated transformation of Oak and Sitka Spruce embryogenic suspension cultures.

4.2 Results

4.2.1 *Agrobacterium*-mediated Transformation of Oak (*Quercus robur* L) Embryogenic Suspension Cultures.

A number of transformation experiments were carried out. The following set of experiments shows the progression from the initial experiment to the final optimized protocol.

4.2.1.1 The effect of Bacterial dilution and incubation time on the efficiency of *Agrobacterium*-mediated transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures

Agrobacterium dilution and incubation time were reported to effect the efficiency of *Agrobacterium*-mediated transformation (Howe *et al.*, 1994; Drake *et al.*, 1997). The following experiment was carried out to assess the optimal *Agrobacterium* dilution and incubation time.

Flasks of 7d old Oak embryogenic suspension cultures were removed from the growth room and sieved through sterile 100 micron mesh sieves. The sieve retentate was weighed and 8 g of cells was placed into sterile 250 cm^3 Erlenmeyer flasks. From overnight cultures of *Agrobacterium tumefaciens* LBA4404:pBI121 at an OD_{600} of 0.8-1.1, 10%, 20% and 50% dilutions were prepared with the culture filtrate up to 100 cm^3. Each of the three flasks of embryogenic suspension cultures was inoculated with one of the Bacterial dilutions. Embryogenic cell samples (5 cm^3) were removed following 60min, 120min, 180min, 240min and 300min from each dilution. Samples were transferred to

sterile 20 cm^3 plastic Universal bottles and centrifuged for 10min at 3,500 x *g*. The supernatant was removed and discarded and the cells resuspended in 5 cm^3 of MS medium minus antibiotics. Samples were clearly labelled with bacterial dilution and incubation time and cultured in the growth room on a gryratory shaker for 72h. All samples were assayed for the presence of β-glucuronidase and the number of *GUS* loci per 1.0 g of cells recorded. Control transformations were set up in exactly the same manner with *Agrobacterium tumefaciens* LBA4404 that did not contain the pBI121 binary vector.

Figure 4.1 outlines the number of *GUS* positive loci per gram of cells transformed with varying bacterial dilutions (10%-50%) and incubated for incubation times varying from 60min-300min. Statistical analysis (Table 4.1, 4.2) revealed that bacterial dilutions of 50% and 20% were significantly different to 10% dilution but not significantly different to each other at the 0.05 level of significance for Tukey's-HSD test. An incubation time of 240 min was found to be significantly different to all other incubation times at the 0.05 level of significance for Tukey's-HSD test. The highest number of *GUS* loci per gram of cells was recorded at 284 for bacterial dilution 50% and incubation time 240min. All control transformations failed to stain blue for all β-glucuronidase assays.

Incubation time (min)	No. of *GUS* loci per sample (1 g fr. wt.)
60	154.40 ± 20.14 **a**
120	232.00 ± 27.44 **a**
180	233.40 ± 19.51 **a**
240	284.40 ± 13.59 **b**
300	242.75 ± 34.42 **a**

Table 4.1. Mean number of *GUS* loci per Oak embryogenic suspension cell sample (1 g fr. wt.) ± SE following inoculation with *Agrobacterium tumefaciens* LBA4404::pBI121 for various time periods. Bacterial dilution 50%, 0 μM acetosyringone, 72h co-cultivation. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test).

Bacterial dilution (% bacteria)	No. of *GUS* loci per sample (1 g fr. wt.)
10	146.80 ± 33.27 **a**
20	207.20 ± 26.07**b**
50	284.40 ± 13.59 **b**

Table 4.2. Mean number of *GUS* loci per Oak embryogenic suspension cell sample (1 g fr. wt.) ± SE following inoculation with different dilutions of *Agrobacterium tumefaciens* LBA4404::pBI121. Incubation time 240 min, acetosyringone concentration 0 μM, co-cultivation time 72h. Means sharing the same letter are not significantly different at the 0.05 level of significance (Tukey's-HSD test).

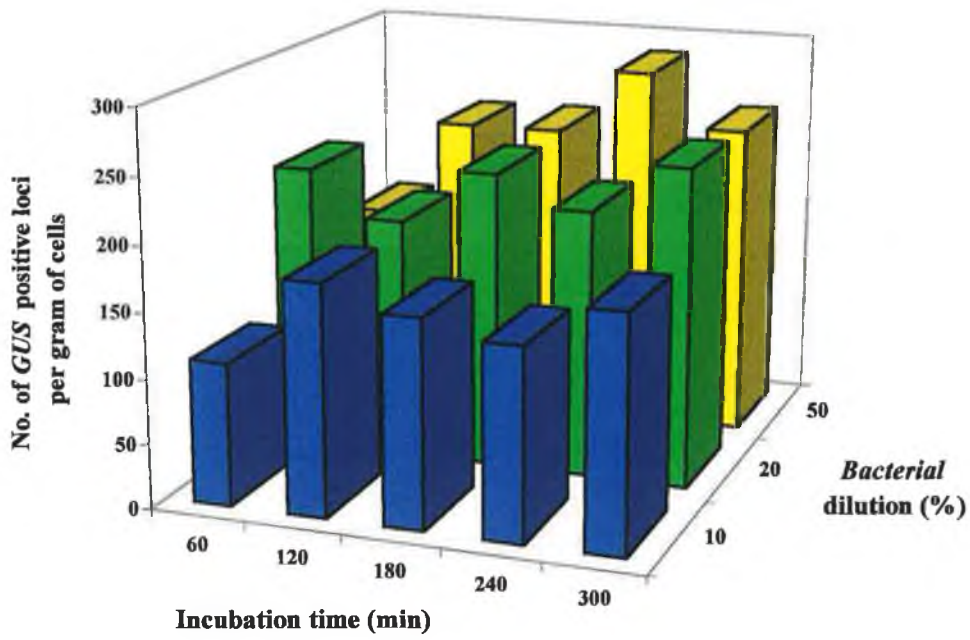


Figure 4.1

Transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures. Incubation times (60-300 min) and bacterial dilutions (10-50%) varied. Acetosyringone was absent and co-cultivation time was set at 72h. Results were taken from 3 cell samples per parameter and the number of *GUS* positive loci were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

4.2.1.2 The effect of co-cultivation time on the efficiency of *Agrobacterium*-mediated transformation of Oak (*Quercus robur* L) embryogenic suspension cultures.

The co-cultivation time of plant cells in residual *Agrobacterium* following transformation has a significant effect on the efficiency of transformation (Drake *et al.*, 1997; Huang *et al.*, 1991; Howe *et al.*, 1994). With Bacterial dilution and incubation time examined the following experiment focuses on the optimum co-cultivation time.

Based on the results from the previous experiment, transformations were set up as above with an bacterial dilution of 50% and an incubation time of 240min. Embryogenic cell samples were co-cultivated for 24h, 48h, 72h and 96h. Samples were assayed as outlined above and results recorded. Again controls were set up with *Agrobacterium tumefaciens* LBA4404 strain.

Figure 4.2 shows the effects of increasing co-cultivation time on the number of *GUS* loci per gram of cells. Results showed that the highest number of *GUS* loci was recorded for a co-cultivation time of 72h. ANOVA analysis showed that the effect of co-cultivation time on the number of *GUS* loci per gram of cells was very highly significant ($p < 0.005$). Tukey's HSD test shows that co-cultivation times of 48h and 72h are not significantly different from each other but are significantly different from all other co-cultivation times (Table 4.3). All control transformations failed to stain blue for all β-glucuronidase assays.

SPOTS

Tukey B[a]

CO	N	Subset for alpha = .05		
		1	2	3
4.00	6	99.3333		
1.00	6		143.6667	
2.00	6			224.3333
3.00	6			252.8333

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 4.3 Tukey's-HSD statistical analysis for the effect of co-cultivation time on the No. of *GUS* loci per gram of Oak embryogenic suspension cultures.

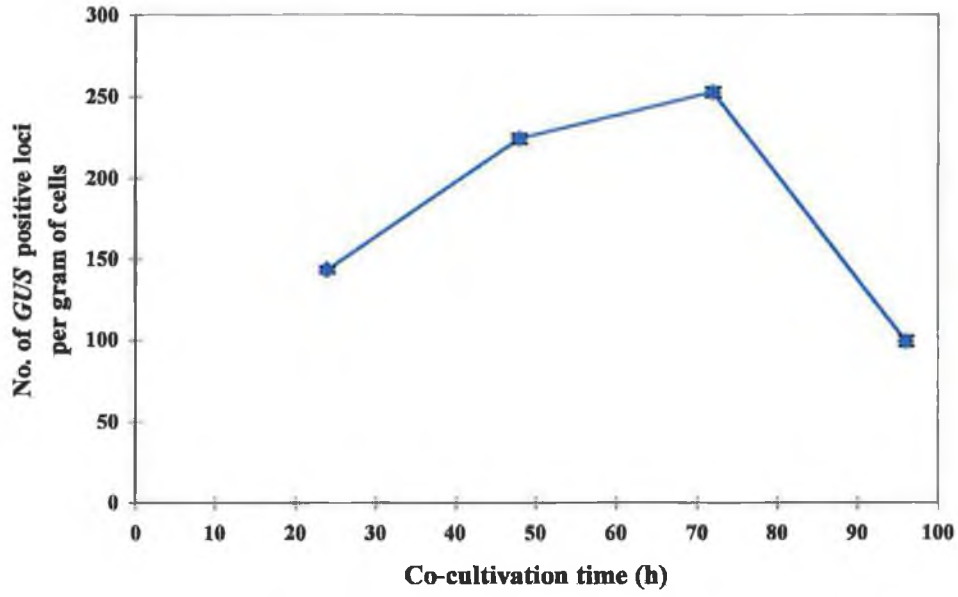


Figure 4.2

The effect of co-cultivation time following *Agrobacterium*-mediated transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures in a 50% dilution of *Agrobacterium* for 240 min. Results were recorded from 3 cell samples per co-cultivation time and numbers of *GUS* positive loci recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate and mean standard error bars are included.

4.2.1.3 The effect of acetosyringone concentration on the efficiency of *Agrobacterium*-mediated transformation of Oak (*Quercus robur* L.) embryogenic supension cultures.

The effect of acetosyringone concentration on transformation efficiency needed to be examined as acetosyringone has been reported to be an inducer of the expression of the *vir* genes. James *et al.*, 1993 reported a significant increase in *GUS* activity in leaf discs of apple with the addition of acetosyringone (0.1mM).

Transformations were set up as previously outlined with the exception that overnight cultures of LBA4404::pBI121 were cultured in Nutrient broth (50 $mgdm^{-3}$ kanamycin) plus 0 μM, 50 μM and 100 μM acetosyringone. Bacterial dilutions of 10%, 20%, 30%, 50% and 100% were used and incubation times of 120 min, 180 min and 240 min for each dilution and acetosyringone concentration. Samples were co-cultivated in the residual *Agrobacterium* for 72h and assayed as with previous experiments. Control experiments were set up as above with LBA4404 *Agrobacterium* strain.

The effects of varying acetosyringone concentration on the number of *GUS* loci per gram of cells is shown in Fig. 4.3 (0 μM), Fig. 4.4 (50 μM) and Fig. 4.5 (100 μM). Statistical analysis by ANOVA showed no significant difference between acetosyringone concentrations ($p=0.325$). Results for all acetosyringone concentrations (Table 4.4, 4.5) revealed that an bacterial dilution of 50% was significantly different to all other dilutions at the 0.05 level of significance for Tukey's-HSD test. Incubation times of 120min and 180min were not significantly different from each other but significantly different to 240min at the 0.05 level of significance for Tukey's-HSD test. All control transformations failed to stain blue for all β-glucuronidase assays.

Bacterial dilution (% bacteria)	No. of *GUS* loci per sample (1 g fr. wt.)
10	122.96 **a**
20	126.32 **a**
30	129.48 **a**
50	206.81**b**
100	167.56 **a**

Table 4.4 Mean number of *GUS* loci per Oak embryogenic suspension cell sample (1 g fr. wt.) following inoculation with various Bacterial dilutions. Incubation time 120min, average acetosyringone concentration of 0 μM, 50 μM, 100 μM and co-cultivation time 72h. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test). The results are taken from an average of all acetosyringone concentrations.

Incubation time (min)	No. of *GUS* loci per sample (1 g fr. wt.)
120	174.68 **a**
180	169.35**a**
240	109.76 **b**

Table 4.5 Mean number of *GUS* loci per Oak embryogenic suspension cell sample (1 g fr. wt.) following inoculation with *Agrobacterium tumefaciens* LBA4404::pBI121 with various incubation times. Bacterial dilution 50%, average acetosyringone concentration of 0 μM, 50 μM, 100 μM, co-cultivation time 72h. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test). The results are taken from an average of all acetosyringone concentrations.

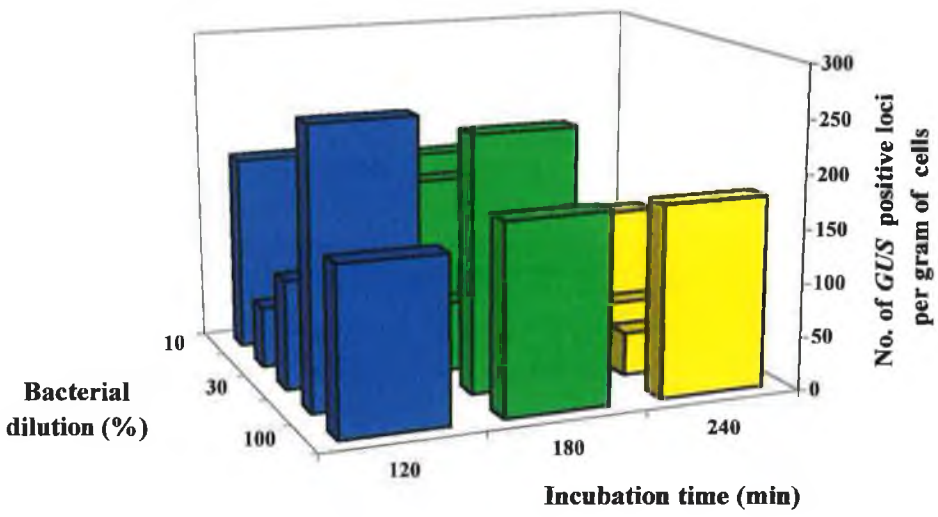


Figure 4.3

Transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures in different bacterial dilutions (10-100%) and different incubation times (120-240min) without acetosyringone and a 72h co-cultivation period. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci per gram of cells were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

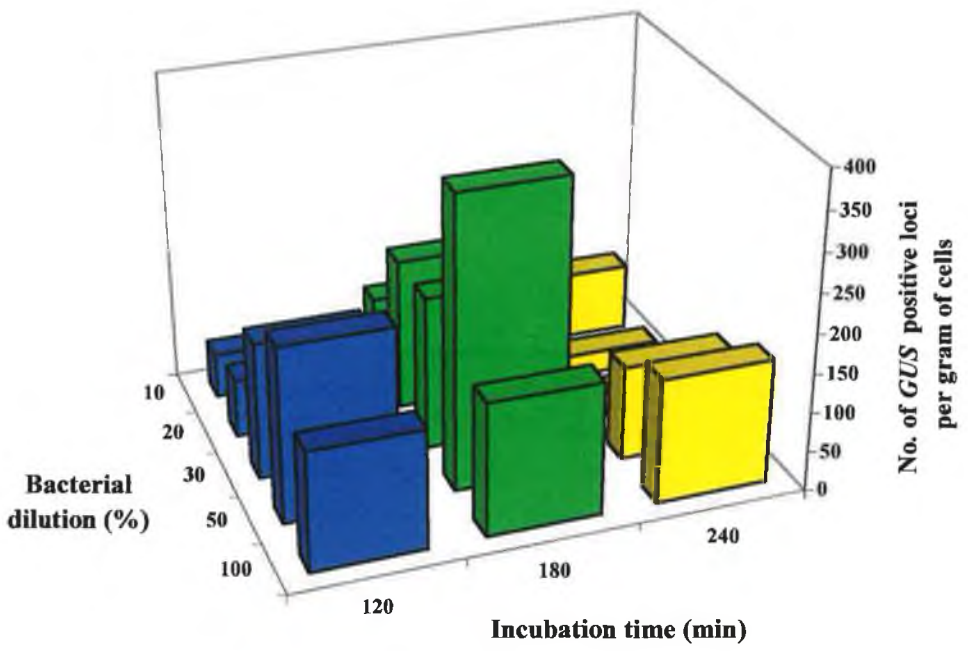


Figure 4.4

Transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures in different bacterial dilutions (10-100%) and different incubation times (120-240min) with 50 μM acetosyringone and a 72h co-cultivation period. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci per gram of cells were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

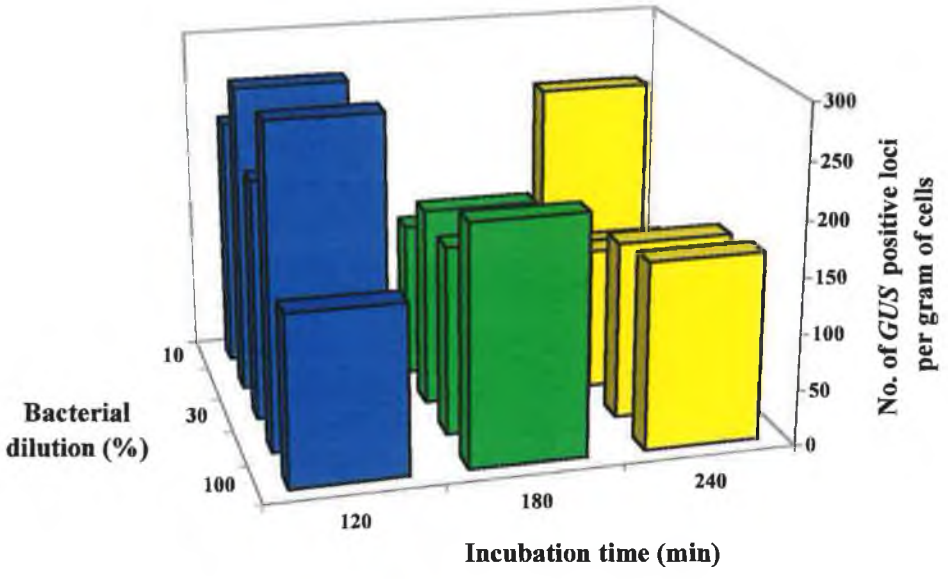


Figure 4.5

Transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures in different bacterial dilutions (10-100%) and different incubation times (120-240min) with 100 μM acetosyringone and a 72h co-cultivation period. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci per gram of cells were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

4.2.1.4 The effect of acetosyringone concentration on the efficiency of *Agrobacterium*-mediated transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures (experiment 2)

The results of the previous experiment did not show significant effects of acetosyringone concentration. The following experiment examines the affects of a larger number of acetosyringone concentrations.

Experiments were designed as above with an bacterial dilution of 50%, incubation time of 180min and a co-cultivation time of 72h. *Agrobacterium tumefaciens* strains LBA4404::pBI121 and LBA4404 were cultured in acetosyringone concentrations of 0 μM, 10 μM, 20 μM, 30 μM, 40 μM. Samples were assayed as above and number of *GUS* loci per sample (1g. fr.wt) were recorded.

Figure 4.6 shows the effect of acetosyringone concentration on the number of *GUS* loci per gram of cells. ANOVA results revealed that the effect of acetosyringone concentration on the number of *GUS* loci per gram of cells was highly significant ($p<0.05$). Tukey's HSD test (Table 4.6) reveals that an acetosyringone concentration of 20 μM results in the highest number of *GUS* loci per gram of cells and is significantly different from all other concentrations. All control transformations failed to stain blue for all β-glucuronidase assays.

SPOTS

Tukey B[a]

ACETO	N	Subset for alpha = .05			
		1	2	3	4
1.00	6	55.8333			
5.00	6	58.4167			
2.00	6		99.0217		
4.00	6			222.1667	
3.00	6				420.0000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 4.6 Tukey's-HSD statistical analysis of the effect of acetosyringone on the No. of *GUS* loci per gram of cells of Oak (*Quercus robur* L.) embryogenic suspension cultures.

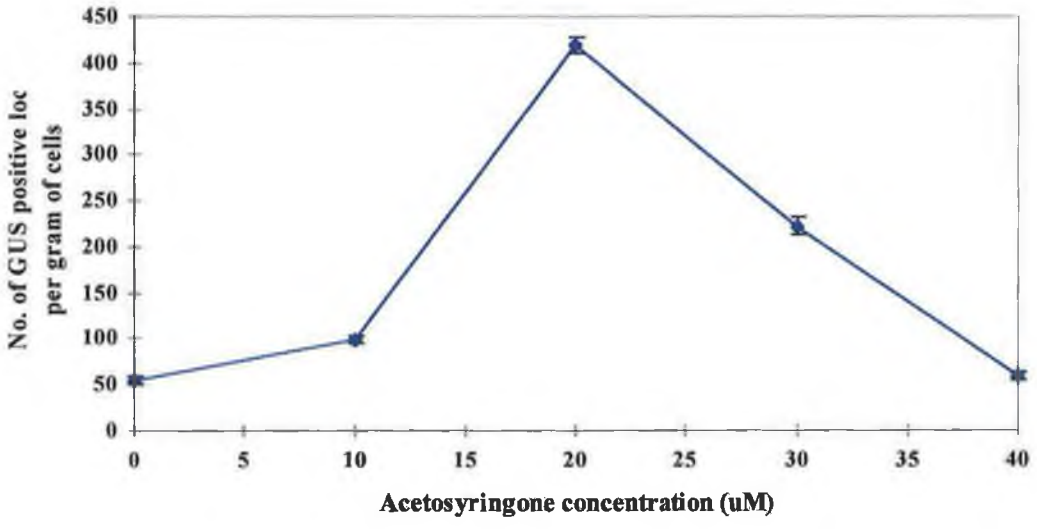


Figure 4.6

The effect of acetosyringone concentration on the No. of *GUS* loci per gram of Oak embryogenic suspension cultures. Bacterial dilution (50%), incubation time (180min) and co-cultivation time (72h). Results were taken from the average of 3 cell samples per concentration and the experiment was carried out in duplicate. Mean standard error bars are included.

4.2.1.5 Optimization of *Agrobacterium*-mediated transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures

An optimum acetosyringone concentration of 25 μM was estimated as the intensity of staining of the embryogenic heads was higher at 30 μM acetosyringone although the number of *GUS* loci per gram of cells was higher at 20 μM. An optimum co-cultivation time (72h) has been achieved and therefore a final examination of bacterial dilutions and incubation times is required as previous results were inconclusive.

Transformations were set up with bacterial dilutions of 10%, 20%, 30%, 50% and 100% with incubation times of 10min, 30min, 60min, 120min, 180min and 240min. Optimum acetosyringone concentration (25 μM) and co-cultivation times (72h) were used. All samples were assayed as above and the numbers of *GUS* loci per cell sample recorded. Control experiments were carried out with *Agrobacterium* strain LBA4404.

Results of the experiment to assess bacterial dilution and incubation time are outlined in Fig. 4.7. Statistical analysis (Table 4.7) using Tukey's-HSD test revealed that the frequency of *GUS* loci per gram of cells at a 50% bacterial dilution was significantly different from all other dilutions at the 0.05 level of significance. An incubation time of 180min was found to be significantly different at a bacterial dilution of 50% to all other incubation times (Table 4.8) at the 0.05 level of significance with an acetosyringone concentration of 25 μM and a co-cultivation time of 72h. All control transformations failed to stain blue for all β-glucuronidase assays.

Bacterial dilution (% bacteria)	No. of *GUS* loci per sample sample (1 g fr. wt.)
10	86.75 ± 35.81 **a**
20	162.50 ± 26.00 **a**
30	448.75 ± 205.94 **a**
50	627.50 ± 139.70 **b**
100	113.75 ± 45.96 **a**

Table 4.7 Mean number of *GUS* loci per Oak embryogenic suspension cell sample (1 g fr. wt.) ± SE following inoculation with different bacterial dilutions. Incubation time 180min, acetosyringone concentration 25 μM, co-cultivation time 72h. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test).

Incubation time (min)	No. of *GUS* loci per sample (1 g fr. wt.)
10	92.53 ± 86.92 **a**
30	383.50 ± 13.85 **a**
60	543.75 ± 244.96 **a**
120	467.25 ± 152.82 **a**
180	627.05 ± 139.70 **b**
240	424.75 ± 83.01 **a**

Table 4.8. Mean number of *GUS* loci per Oak embryogenic suspension cell sample (1 g fr. wt.) ± SE following inoculation with *Agrobacterium tumefaciens* LBA4404::pBI121 with various incubation times. Bacterial dilution 50%, acetosyringone concentration 25μM, co-cultivation time 72h. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test).

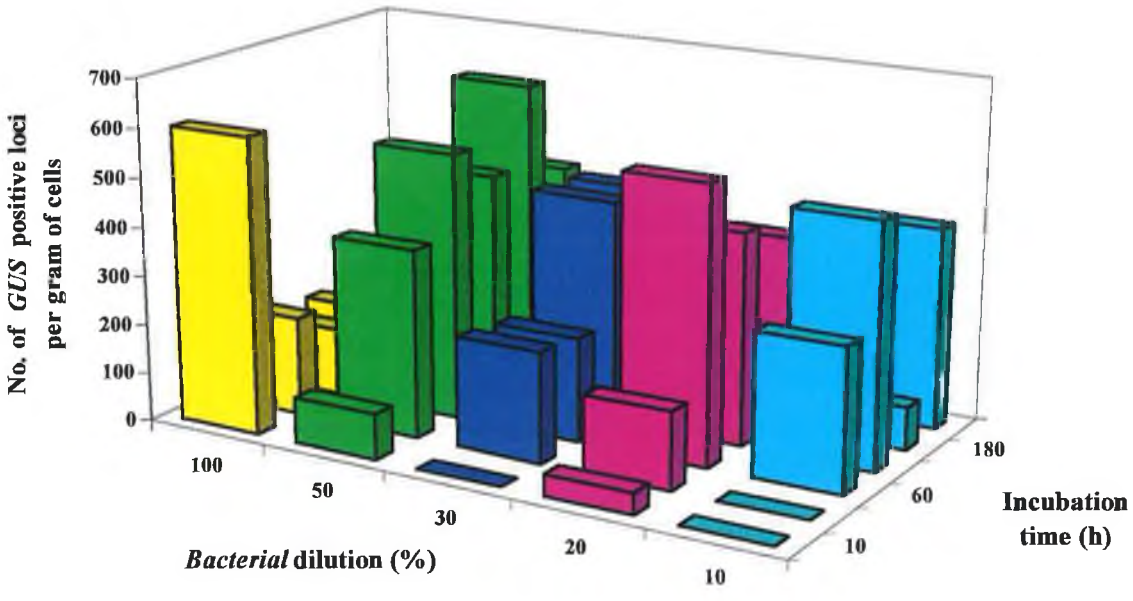


Figure 4.7

Number of *GUS* positive loci per gram of Oak (*Quercus robur* L.) embryogenic suspension cultures in different bacterial dilutions (10-100%) and incubation times (10-180min). Co-cultivation time of 72h and acetosyringone concentration of 25 μM. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci per gram of cells recorded following β-glucuronidase histochemical assays. The experiment was carried out in duplicate.

4.2.1.6 Transient transformation of Oak (*Quercus robur* L.) embryogenic suspension cultures

With optimum values for Bacterial dilution, incubation time, acetosyringone concentration and co-cultivation time evaluated an experiment was carried out where transformed samples were allowed to grow beyond the 72h co-cultivation period.

Transformations were set up with an acetosyringone concentration of 25 μM and a bacterial dilution of 50% and incubated for 120min. After infection with *Agrobacterium* the cells were then sieved through 100 micron mesh sieves, washed twice in sterile distilled water and transferred to 50 cm^3 of MS minus antibiotics in 250 cm^3 Erlenmeyer flasks. Flasks were cultured in the growth room on a gyratory shaker at 100 rpm for 72h. Following the 72h co-cultivation period samples were aseptically taken and assayed for β-glucuronidase activity. To the remaining sample 500 $mgdm^{-3}$ cefotaxime was filter sterilised and it was replaced in the growth room. Paromomycin (30 $mgdm^{-3}$) was filter sterilised into the transformations 72h later and another sample taken for assay. Samples were then taken every 5d for assay and transformations subcultured every 10d into MS (500 $mgdm^{-3}$ cefotaxime and 30 $mgdm^{-3}$ paromomycin) liquid medium.

Plate 4.1 shows Oak embryogenic callus which had been transformed for 6 months. The embryogenic suspension culture continued to express β-glucuronidase activity. Oak embryogenic suspension cultures expressed β-glucuronidase activity for up to 1 yr in the laboratory. All control transformations failed to stain blue for all β-glucuronidase assays.

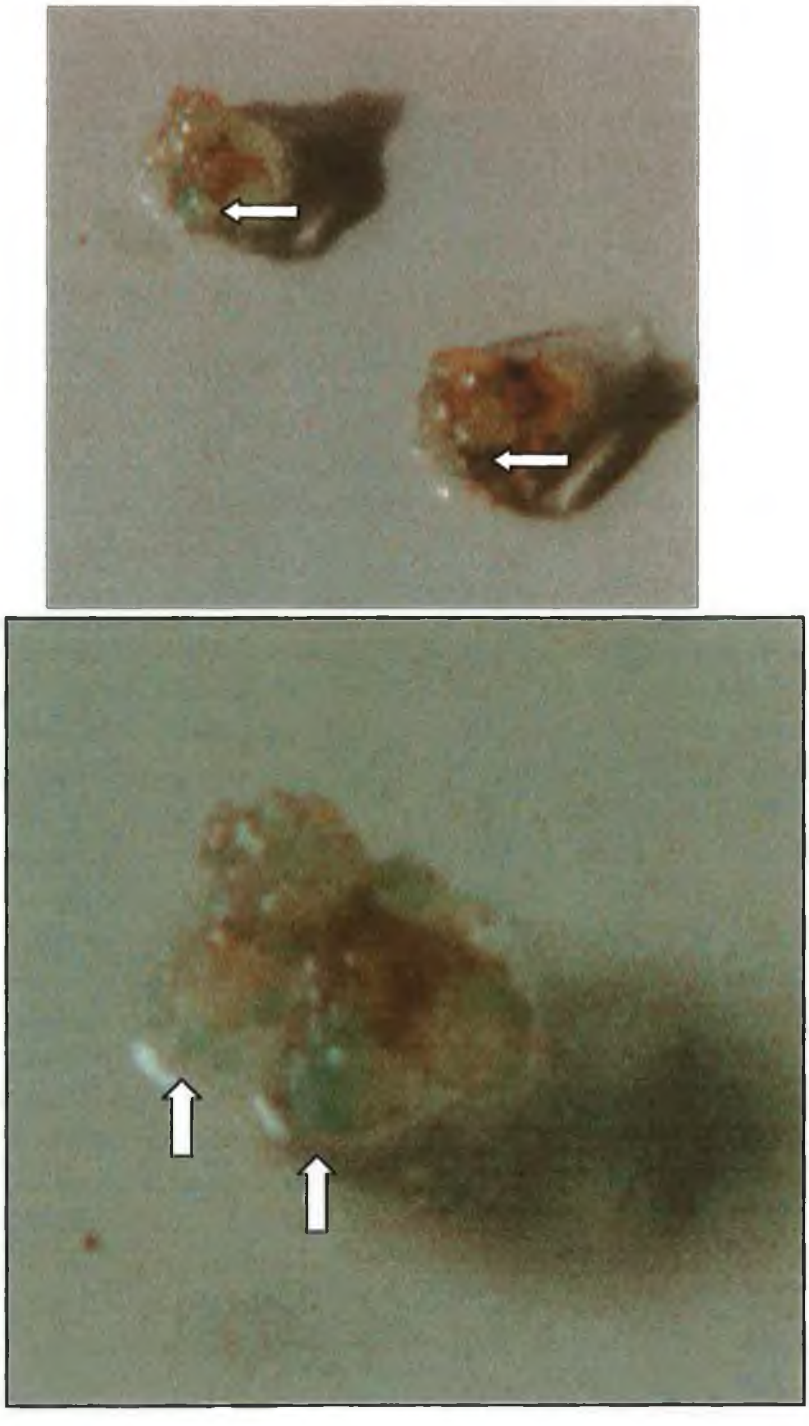


Plate 4.1

Transiently *Agrobacterium*-mediated transformed Oak (*Quercus robur* L.) embryogenic suspension cultures. Bacterial dilution (50%), incubation time (180min), acetosyringone concentration (25 μM) and co-cultivation time (72h). The arrows indicate areas of *GUS* positive loci due to cleavage of X Gluc with the production of a blue colouration. Non-blue areas were non-transformed sections.

4.2.2 *Agrobacterium*-mediated Transformation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Embryonal Suspensor Masses

A number of transformation experiments were carried out. The following set of experiments shows the progression from the initial experiment to the final optimized protocol.

4.2.2.1 The effects of bacterial dilution and incubation time on the efficiency of *Agrobacterium*-mediated transformation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses

Optimized protocols for Oak transformation led us to believe that transient transformation was achievable with Sitka Spruce ESMs in suspension culture. The following experiment was carried out to assess the optimal *Agrobacterium* dilution and incubation time.

Flasks of 7d old Sitka Spruce embryonal suspensor mass liquid cultures were removed from the growth room and sieved through sterile 100 micron mesh sieves. The sieve retentate was weighed and into sterile 250 cm^3 Erlenmeyer flasks approximately 8 g of cells were placed. From overnight cultures of *Agrobacterium tumefaciens* LBA4404:pBI121 at an OD_{600} of 0.8-1.1 10%, 20% and 50% dilutions were prepared with the culture filtrate up to 100 cm^3. Each of the three flasks of cells was inoculated with one of the bacterial dilutions. Culture samples (5 cm^3) were removed following 10min, 30min, 60min and 120min from each dilution. Samples were transferred to sterile 20 cm^3 plastic Universal bottles and centrifuged for 10min at 3,500 x *g*. The supernatant was removed and discarded and the cells resuspended in 5 cm^3 of Sitka Spruce embryogenesis, embryo

initiation medium minus antibiotics. Samples were clearly labelled and cultured in the growth room on a gyratory shaker for 72h. All samples were assayed for the presence of β-glucuronidase and the number of *GUS* loci per gram of cells recorded. Control transformations were set up in exactly the same manner with *Agrobacterium tumefaciens* LBA4404 which did not contain the pBI121 binary vector.

Figure 4.8 shows the effect of Bacterial dilution (10%-50%) and incubation time (10min-120min) on the number of *GUS* loci per gram of Sitka Spruce embryonal suspensor masses. Statistical analysis (Table 4.9) revealed using Tukey's-HSD test at the 0.05 level of significance that a bacterial dilution of 50% was significantly different to all other dilutions at 10 min incubation time. However results showed that for incubation time no two times were significantly different from each other at the 0.05 level of significance at a 50 % bacterial dilution. The highest number of *GUS* loci per gram was 2618 loci for an Bacterial dilution of 50% and an incubation time of 60min. All control transformations failed to stain blue for all β-glucuronidase assays.

Bacterial dilution (% bacteria)	No. of *GUS* loci per sample (1 g fr. wt.)
10	188.75 ± 128.22 **a**
20	963.50 ± 523.51 **a**
50	1807.50 ± 288.62 **b**

Table 4.9. Mean number of *GUS* loci per Sitka Spruce embryonal suspensor mass cell sample (1 g fr. wt.) ± SE following inoculation with different dilutions of *Agrobacterium tumefaciens* LBA4404::pBI121. Incubation time 10 min, acetosyringone concentration 0 μM, co-cultivation time 72h. Means sharing the same letter are not significantly different at the 0.05 level of significance (Tukey's-HSD test).

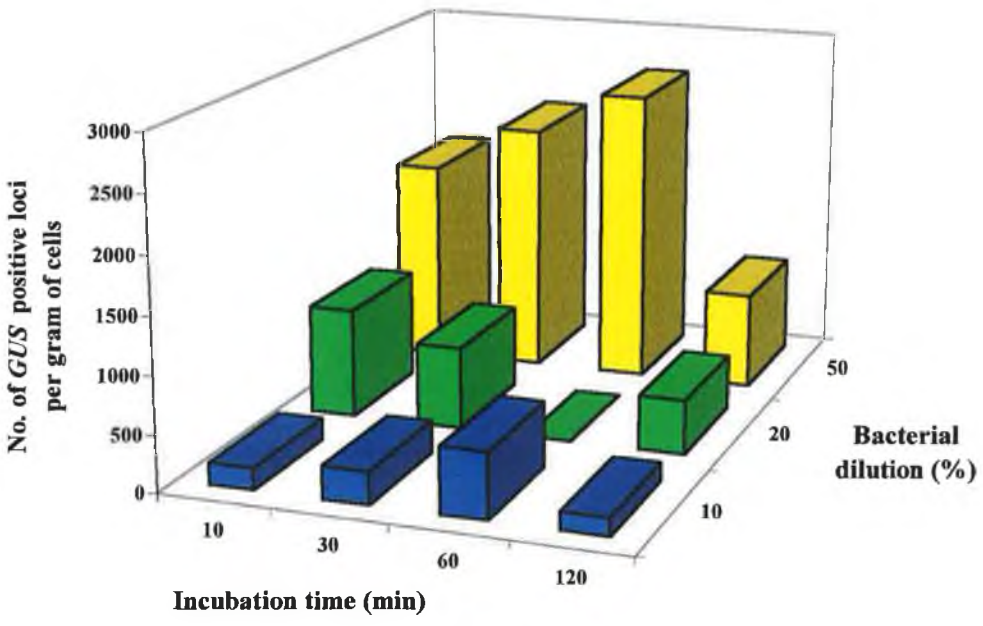


Figure 4.8

Number of *GUS* positive loci per gram of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass. Incubation times (10-120min) and bacterial dilutions (10-50%) varied. Acetosyringone was absent and a set co-cultivation time of 72h was used. Results were calculated from 3 cell samples per parameter and the number of *GUS* positive loci were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

4.2.2.2 The effect of co-cultivation time on the efficiency of *Agrobacterium*-mediated transformation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses

With bacterial dilution and incubation time examined the following experiment focuses on the optimum co-cultivation time.

Based on the results from the previous experiment transformations were set up as above with an bacterial dilution of 50% and an incubation time of 60min. Cell samples were co-cultivated for 24h, 48h, 72h and 96h in the residual *Agrobacterium*. Samples were assayed as outlined above and results recorded. Again controls were set up with *Agrobacterium tumefaciens* LBA4404 strain.

Figure 4.9 outlines the effect of increasing the co-cultivation time on the number of *GUS* loci per gram of cells. The highest number of *GUS* loci was recorded for a 72h co-cultivation period (Table 4.10) and was significantly different to all other co-cultivation times. ANOVA results showed that the effect of co-cultivation time on the number of *GUS* loci per gram of cells was highly significant ($p<0.05$). All control transformations failed to stain blue for all β-glucuronidase assays.

GUS

Tukey B[a]

CO	N	Subset for alpha = .05		
		1	2	3
1.00	6	122.5000		
2.00	6	164.1667		
4.00	6		592.1667	
3.00	6			2557.1667

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 4.10 Tukey's HSD statistical analysis for the effect of co-cultivation time on the No. of *GUS* positive loci per gram of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass.

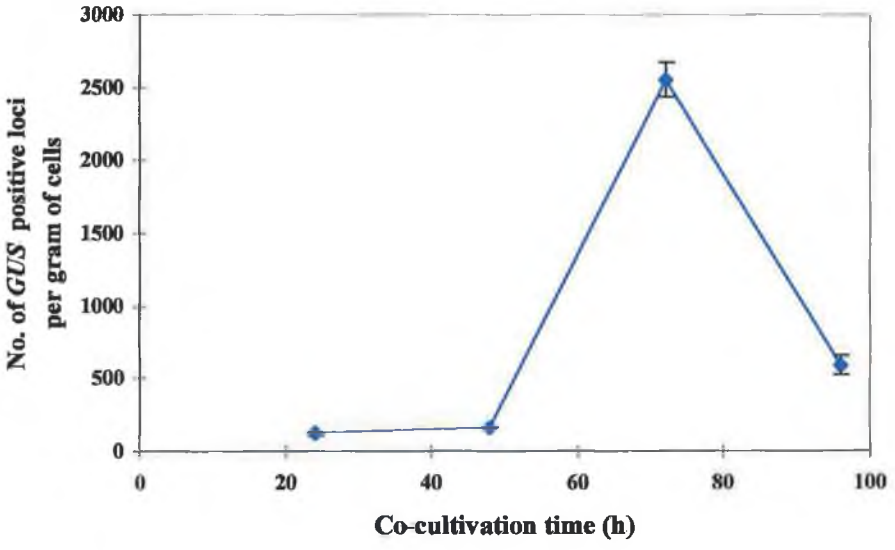


Figure 4.9

The relationship between co-cultivation time and the No. of *GUS* positive loci per gram of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor mass in a 50% dilution of *Agrobacterium* for 60 min. Results were calculated from 3 cell samples per co-cultivation time and numbers of *GUS* positive loci were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate and mean standard error bars are included.

4.2.2.3 The effect of acetosyringone concentration on the efficiency of *Agrobacterium*-mediated transformation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses

The effect of acetosyringone concentration on transformation efficiency needed to be examined. Drake *et al.*, 1997 reported greater *Agrobacterium*-mediated transformation efficiency with 50 μM acetosyringone in ESM solid medium cultures of Sitka Spruce.

Transformations were set up as previously outlined with the exception that overnight cultures of LBA4404::pBI121 were cultured in Nutrient broth (50 $mgdm^{-3}$ kanamycin) at three different concentrations of acetosyringone 0 μM, 50 μM and 100 μM. Bacterial dilutions of 10%, 20% and 50% were used and incubation times of 10 min, 30 min and 60 min for each dilution and acetosyringone concentration. Samples were co-cultivated in the residual *Agrobacterium* for 72h and assayed as with previous experiments. Control experiments were again set up as above with LBA4404 *Agrobacterium* strain.

The effect of increasing acetosyringone concentration on the number of *GUS* loci per gram of cells is outlined in Fig. 4.10 (0 μM), Fig. 4.11 (10 μM), Fig. 4.12 (20 μM) and Fig. 4.13 (50 μM). Statistical analysis (Table 4.11) on all acetosyringone concentrations revealed that the optimum bacterial dilution was 50% as it was significantly greater to all other dilutions at the 0.05 level of significance (Tukey's-HSD). An incubation time of 60min was significantly different to all other times at the 0.05 level of significance for Tukey's-HSD test (Table 4.12). ANOVA analysis however did not reveal a significant effect of acetosyringone concentration ($p=0.305$) at different incubation times and bacterial dilutions. All control transformations failed to stain blue for all β-glucuronidase assays.

Bacterial dilution (% bacteria)	No. of *GUS* loci per sample (1 g fr. wt.)
10	392.75 **a**
20	266.94 **a**
50	897.80 **b**

Table 4.11 Mean number of *GUS* loci per Sitka Spruce embryonal suspensor mass cell sample (1 g fr. wt.) following inoculation with various bacterial dilutions. Incubation time 60min, acetosyringone concentration 0 μM, 50 μM, 100 μM and co-cultivation time 72h. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test).

Incubation time (min)	No. of *GUS* loci per sample (1 g fr. wt.)
10	370.30 **a**
30	365.72 **a**
60	821.47 **b**

Table 4.12. Mean number of *GUS* loci per Sitka Spruce embryonal suspensor mass cell sample (1 g fr. wt.) following inoculation with *Agrobacterium tumefaciens* LBA4404::pBI121 with various incubation times. Bacterial dilution 50%, acetosyringone concentration 0 μM, 50 μM, 100 μM, co-cultivation time 72h. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test).

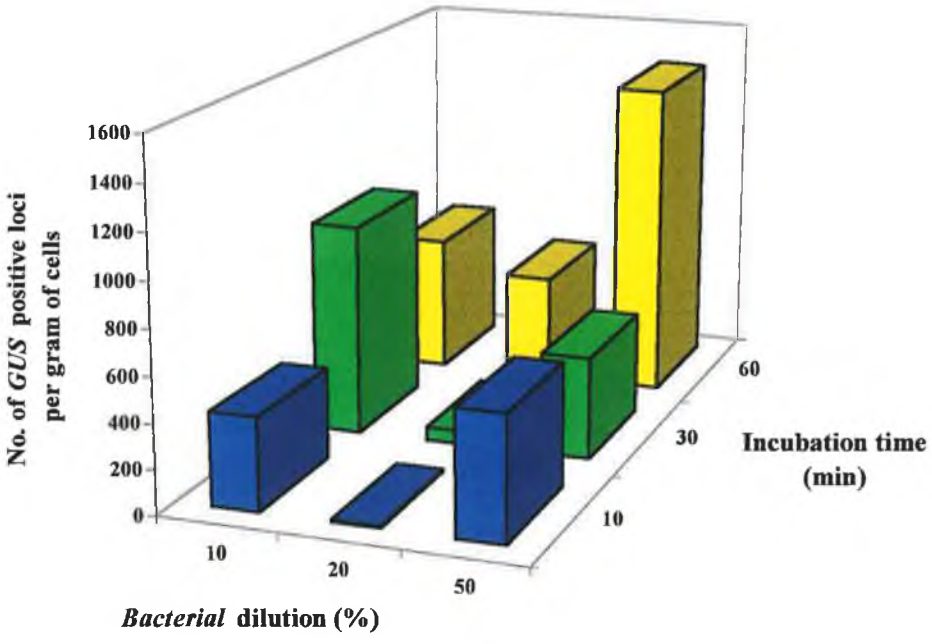


Figure 4.10

The number of *GUS* positive loci per gram of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in different bacterial dilutions (10-50%) and different incubation times (10-60min) in the absence of acetosyringone and a 72h co-cultivation period. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci per gram of cells were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

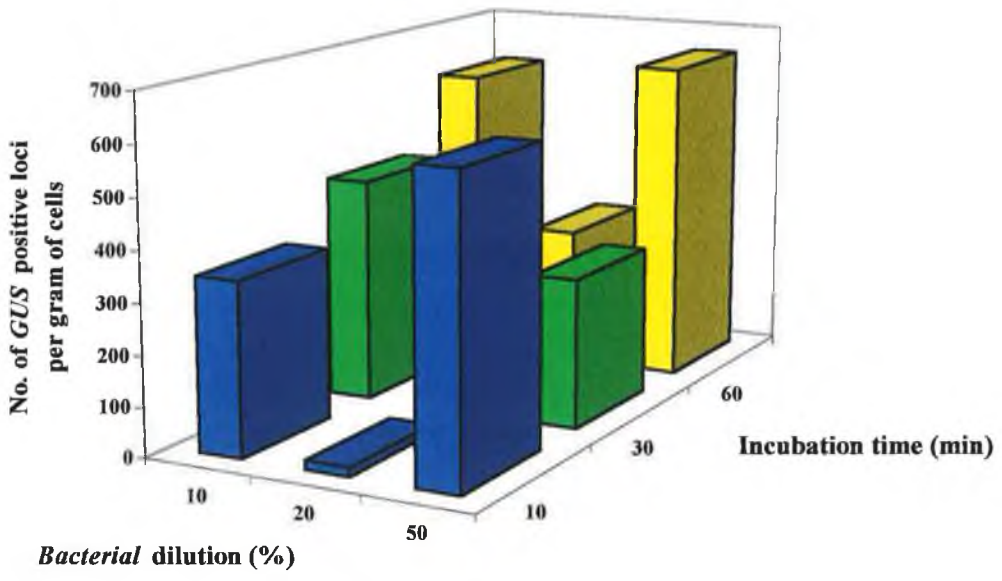


Figure 4.11

The number of *GUS* positive loci per gram of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in different bacterial dilutions (10-50%) and different incubation times (10-60min) with 10 μM acetosyringone and a 72h co-cultivation period. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci per gram of cells were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

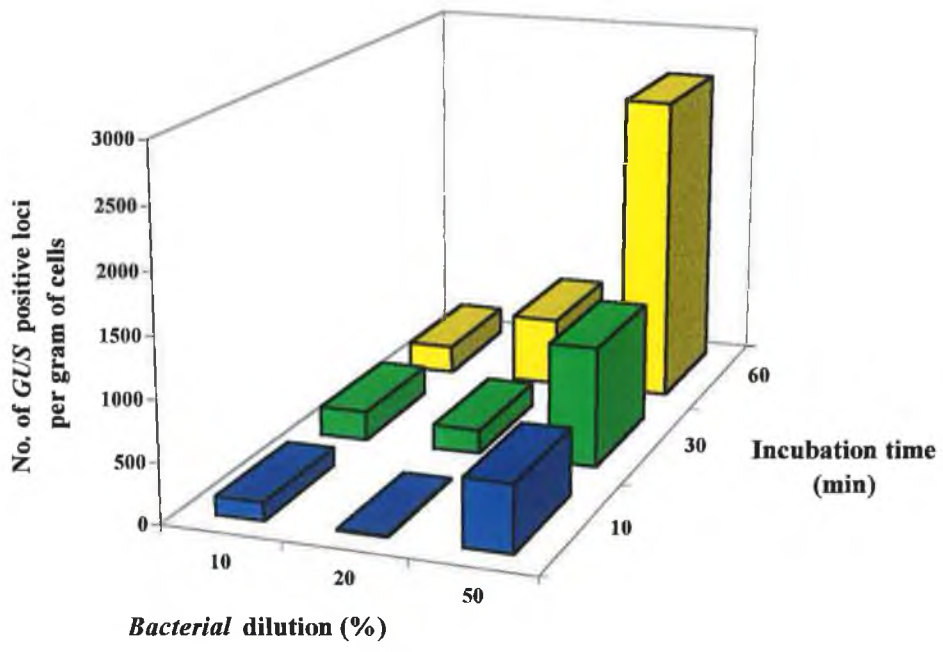


Figure 4.12

The number of *GUS* positive loci per gram of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in different bacterial dilutions (10-50%) and different incubation times (10-60min) with 20 μM acetosyringone and a 72h co-cultivation period. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci per gram of cells were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

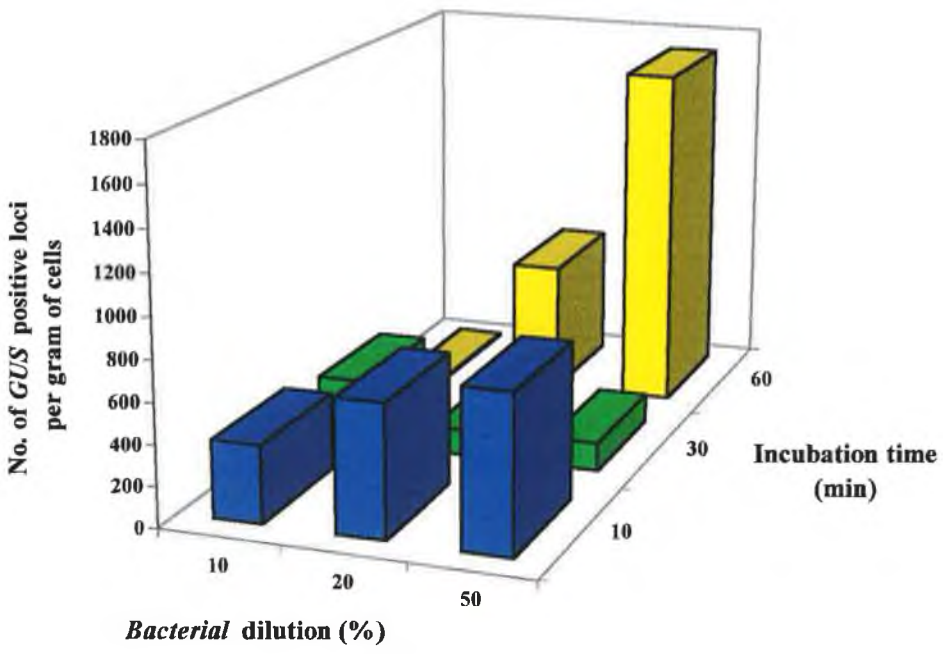


Figure 4.13

Transformation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in different bacterial dilutions (10-50%) and different incubation times (10-60min) with 50 μM acetosyringone and a 72h co-cultivation period. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci per gram of cells were recorded following β-glucuronidase histochemical assays. Experiments were carried out in duplicate.

4.2.2.4 The effect of acetosyringone concentration on the efficiency of *Agrobacterium*-mediated transformation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses (experiment 2)

As the results from the previous experiment did not show significant effects of acetosyringone concentration the following experiment examines the effects of a larger variety of acetosyringone concentrations.

Experiments were designed as above with an bacterial dilution of 50%, incubation time of 60min and a co-cultivation time of 72h. *Agrobacterium tumefaciens* strains LBA4404::pBI121 and LBA4404 were cultured in acetosyringone concentrations of 0 μM, 25 μM, 50 μM, 70 μM, 100 μM and 125 μM. Samples were assayed as above and number of *GUS* loci per sample were recorded.

Figure 4.14 shows the effect of increasing acetosyringone concentration on the number of *GUS* loci per gram of cells. The highest number of *GUS* loci was recorded for 25 μM acetosyringone with a bacterial dilution of 50% and incubation time of 60min (Table 4.13) and Tukey's HSD test showed it to be significantly different to all other concentrations. ANOVA results revealed that the effect of acetosyringone concentration on the number of *GUS* loci per gram of cells was highly significant ($p<0.05$). All control transformations failed to stain blue for all β-glucuronidase assays.

SPOT

Tukey B[a]

ACET	N	Subset for alpha = .05				
		1	2	3	4	5
6.00	6	134.5000				
5.00	6		291.0000			
4.00	6		394.1667	394.1667		
1.00	6			458.6667		
3.00	6				647.5000	
2.00	6					3010.5000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Table 4.13 Tukey's HSD statistical analysis for the effect of acetosyringone concentration on the The number of *GUS* positive loci per gram of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses.

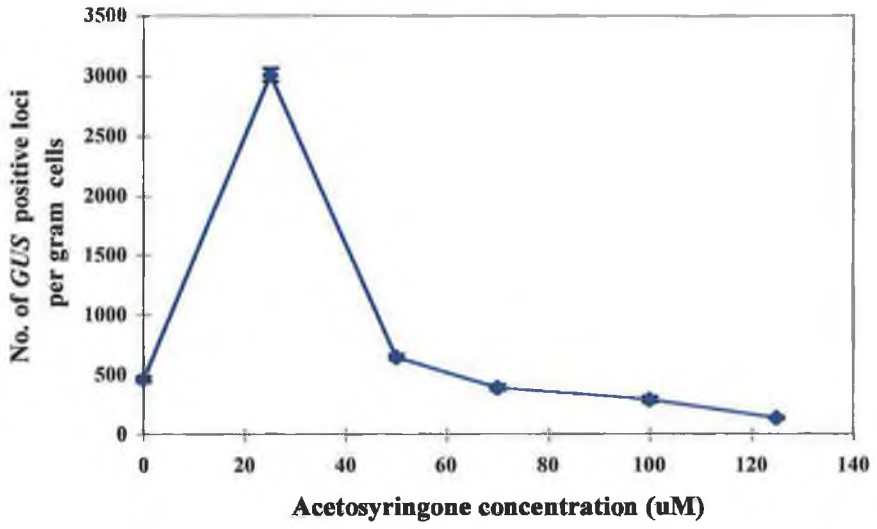


Figure 4.14

The effect of acetosyringone concentration on the number of *GUS* positive loci per gram of Sitka Spruce embryonal suspensor masses. Bacterial dilution (50%), incubation time (60min) and co-cultivation time (72h). Results were taken from the average of 3 cell samples per concentration and the experiment was carried out in duplicate. Mean standard error bars are included.

4.2.2.5 Optimization of *Agrobacterium*-mediated transformation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses

Optimum acetosyringone concentration (25 μM) and co-cultivation time (72h) have been achieved therefore a final examination of bacterial dilutions and incubation times was required.

Transformations were set up with bacterial dilutions of 10%, 20%, 30%, 50% and 100% with incubation times of 10min, 30 min, 60 min and 120min. Optimum acetosyringone concentration and co-cultivation times were used. All samples were assayed as above and the numbers of *GUS* loci per cell sample recorded. Control experiments were carried out with *Agrobacterium* strain LBA4404.

The results of the final experiment to assess the effect of bacterial dilution and incubation time are shown in Fig. 4.15. Statistical analysis using Tukey's-HSD test shows that the optimum bacterial dilution was identified as 50% as it was significantly greater to all other dilutions at an incubation time of 60 min at the 0.05 level of significance (Table 4.14). Results on incubation time show that times of 30min and 60min were significantly greater to all other incubation times but not to each other at the 0.05 level of significance at 50 % bacterial dilution (Tukey's-HSD test) (Table 4.15). All control transformations failed to stain blue for all β-glucuronidase assays.

Bacterial dilution (% bacteria)	No. of *GUS* loci per sample (1 g fr. wt.)
10	669.00 ± 27.36 **a**
20	161.17 ± 16.50 **a**
30	823 ± 10.28 **a**
50	3156.67 ± 105.45 **b**
100	601.50 ± 9.82 **a**

Table 4.14 Mean number of *GUS* loci per Sitka Spruce embryonal suspensor mass cell sample (1 g fr. wt.) ± SE following inoculation with various bacterial dilutions. Incubation time 60min, acetosyringone concentration 25 μM, co-cultivation time 72h. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test).

Incubation time (min)	No. of *GUS* loci per sample (1 g fr. wt.)
10	928.50 ± 10.26 **a**
30	2044.17 ± 85.42 **b**
60	3156.67 ± 105.45 **b**
120	783.67 ± 47.88 **a**

Table 4.15. Mean number of *GUS* loci per Sitka Spruce embryonal suspensor mass cell sample (1 g fr. wt.) ± SE following inoculation with *Agrobacterium tumefaciens* LBA4404::pBI121 with various incubation times. Bacterial dilution 50%, acetosyringone concentration 25μM, co-cultivation time 72h. Means sharing the same letter were not significantly different at the 0.05 level of significance (Tukey's-HSD test).

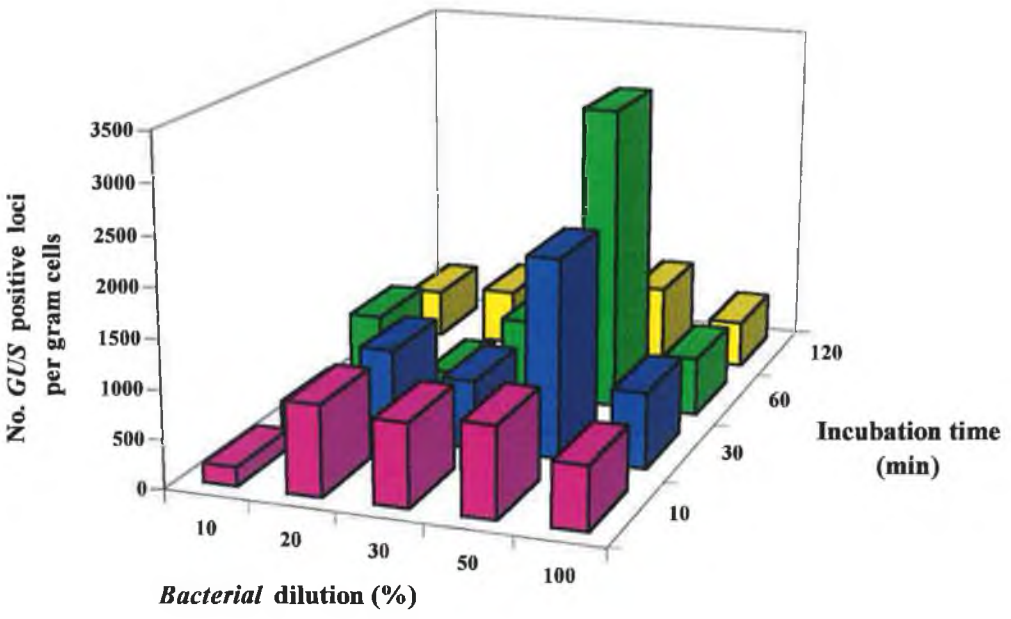


Figure 4.15

Number of *GUS* positive loci per gram of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in different bacterial dilutions (10-100%) and incubation times (10-120min). Co-cultivation time of 72h and acetosyringone concentration of 25 μM. Results were calculated from 3 cell samples per parameter and numbers of *GUS* positive loci recorded following β-glucuronidase histochemical assays. The experiment was carried out in duplicate.

4.2.2.6 Transient *Agrobacterium*-mediated transformation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses

With optimum values for bacterial dilution, incubation time, acetosyringone concentration and co-cultivation time evaluated a final experiment was carried out where transformed samples were allowed to grow beyond the 72h co-cultivation period.

Transformations were set up with an acetosyringone concentration of 25 μM and an bacterial dilution of 50% in 100 cm^3 quantities and incubated for 60min. Transformations were then sieved through 100 micron mesh sieves and washed twice in sterile distilled water and transferred to 50 cm^3 of Sitka Spruce embryogenesis, embryo initiation media minus antibiotics in 250 cm^3 Erlenmeyer flasks. Flasks were cultured in the growth room on a gryratory shaker at 100 rpm for 72h. Following the 72h co-cultivation period samples were aseptically taken and assayed for β-glucuronidase activity. To the remaining sample 500 $mgdm^{-3}$ cefotaxime was filter sterilised and it was replaced in the growth room. Paromomycin (3 $mgdm^{-3}$) was filter sterilised into the transformations 72h later and another sample taken for assay. Samples were then taken every 5d for assay and transformations subcultured every 10d into Sitka Spruce embryogenesis, embryo initiation medium (500 $mgdm^{-3}$ cefotaxime, 3 $mgdm^{-3}$ paromomycin).

Plate 4.2 shows Sitka Spruce embryonal suspensor masses expressing β-glucuronidase activity following transformation with a 50% bacterial dilution, incubation time 60min, 25 μM acetosyringone and a co-cultivation time of 72h. Embryonal suspensor masses had been transformed for 6 months. All control transformations failed to stain blue for all β-glucuronidase assays.

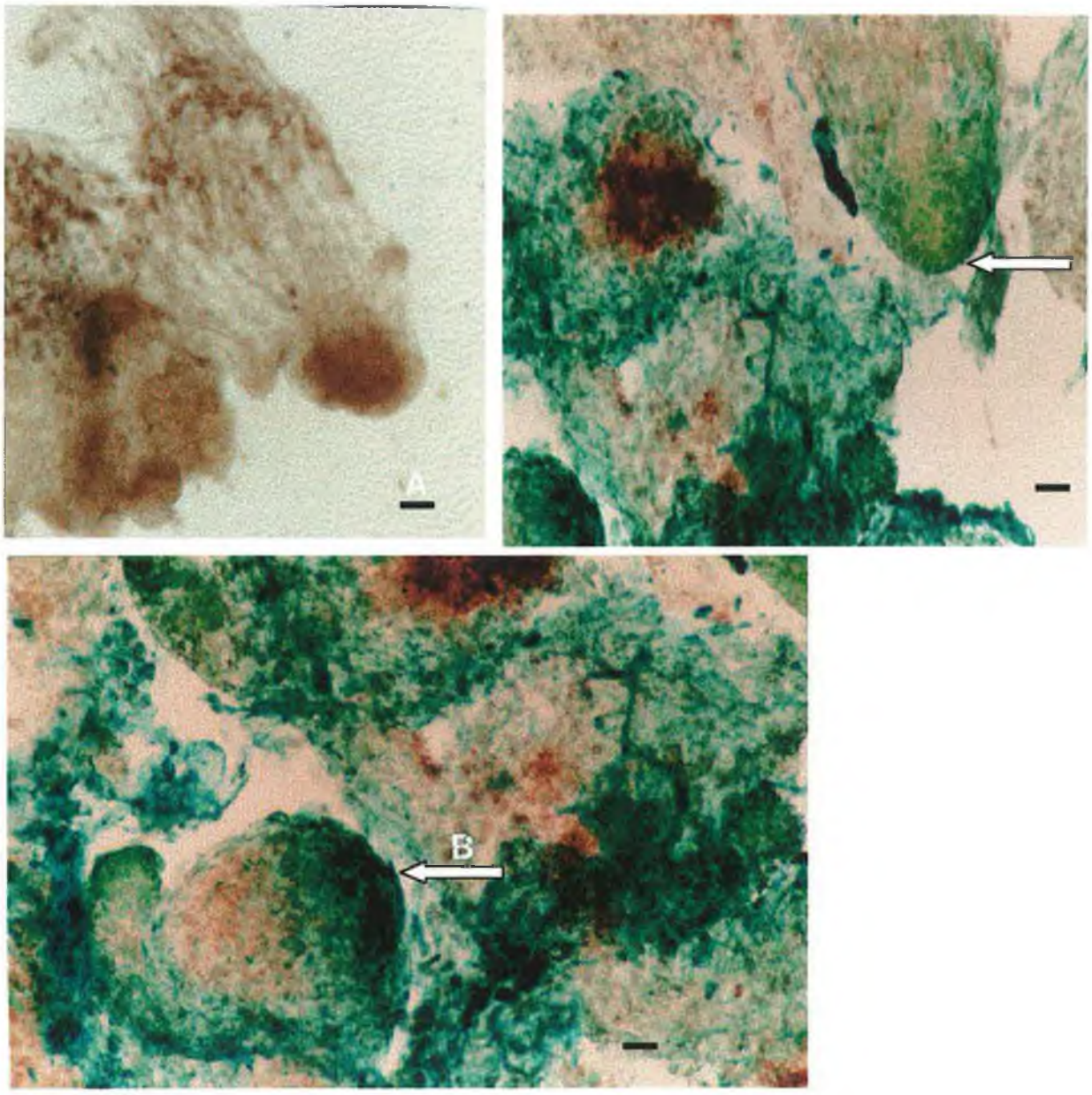


Plate 4.2

Non-transformed (A) and transiently *Agrobacterium*-mediated transformed (B) Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses. The arrow shows *GUS* positive loci due to cleavage of X Gluc with the production of a blue colouration in the embryogenic heads of stage 1 somatic embryos. Bacterial dilution (50%), incubation time (60min), acetosyringone concentration (25 µM) and co-cultivation time (72h). Embryos were examined under 10x magnification on a compound microscope.
Bar=200 µm

4.3 Discussion

Transformation procedures allow one to make small specific changes in the genome of a cell i.e. the addition of one or a few genes. Transformation of plants offers the potential to make relatively quick specific changes without disrupting the plants desirable genetic constitution (Sheurman and Dandekar, 1993). The *Agrobacterium*-mediated gene transfer technique has proven to be the most understood method for introducing a wide variety of genes into plants. Experiments in this chapter were carried out to investigate the efficiency of transformation of embryogenic suspension cultures of Oak and Sitka Spruce were by *Agrobacterium tumefaciens* strain LBA4404::pBI121 containing the binary vector pBI121 (Jefferson, 1987) derived from the vector pBIN19. The binary plasmid contained two chimeric genes, β-glucuronidase (*GUS*) and the *npt* II gene that confers antibiotic resistance.

A number of factors needed to be assessed such as the effects of *Agrobacterium* dilution, incubation time and co-cultivation time on the efficiency of β-glucuronidase expression in transformed cells.

Initial experiments showed that for Oak transformations, bacterial dilutions of 20% and 50% were significantly different to 10%. Later experiments confirmed that a 50% dilution produced the highest number of *GUS* loci per gram. Bacterial dilutions of 50% for Sitka Spruce cells resulted in a 3-fold increase in the number of *GUS* expressing cells compared to a dilution of 30%. Drake *et al.*, 1997 reported similar results for *Agrobacterium*-mediated transformed of Sitka Spruce ESMs on solid medium. An bacterial culture diluted equally with medium produced the highest number of *GUS* positive loci.

The duration of incubation time was shown to have a highly significant effect on the number of cells expressing β-glucuronidase activity in Oak and Sitka Spruce. Roest & Evers, 1991 incubated nodal stem explants of *Quercus robur* L. for 30min before removing them from the bacterial dilution. Our results showed that longer incubation periods were required to reach high levels of expression. Incubation times above 60min proved different to lower incubation times with a 6-fold increase from 10-180 min for Oak embryogenic cultures incubated in a 50% bacterial dilution. Sitka Spruce embryonal suspensor masses required shorter incubation times. Incubation times between 30-60min proved significantly greater to both lower and higher times increasing the number of *GUS* expressing cells over 3-fold in 50% bacterial dilution. The difference between Oak and Sitka Spruce embryogenic cultures may be due to the fact that Sitka cultures are more friable and cells can be accessed more easily. No significant difference was found in the number of *GUS* positive loci of Sitka Spruce ESMs by Drake *et al.*, 1997 over a range of incubation times between 10-120 min. Gonzalez *et al.*, 1998 found that the optimum incubation time for transformation of embryogenic suspension cultures of Cassava in an *Agrobacterium tumefaciens* dilution with 100 μM acetosyringone was 60min.

An optimum co-cultivation time before the elimination of *Agrobacterium* with cefotaxime antibiotic was shown to be 72h for both tree species. An almost 2-fold increase in the number of *GUS* loci was noticed for Oak cells from 24-72h in a 50% dilution for 240min incubation time whereas a larger increase of 20.9-fold was found for Sitka Spruce at 50% dilution and 60min incubation time. Drake *et al.*, 1997 reported a 15-fold increase in *GUS* loci in Sitka Spruce ESMs from 48-72h. A co-cultivation time of 48h proved most optimal for the *Agrobacterium*-mediated transformation of leaf discs of *Populus nigra* (Confalonieri *et al.*, 1994). A 48h co-cultivation period was required for hybrid poplar suspension cultures (Howe *et al.*, 1994). Howe *et al.*, failed to recover any transformants

after 24h. Co-cultivation periods greater than 4-5d are not recommended due to increasing difficulty in eliminating the *Agrobacterium*. A recent study by Hammerschlag *et al.*, 1997 suggested that vacuum infiltration of explants with a low pH medium followed by short exposure to high levels of cefotaxime could be effective in eliminating *Agrobacterium tumefaciens* from explants following prolonged co-cultivation.

Acetosyringone is a naturally occurring wound response phenolic and it has been identified as a plant signal molecule that induces the activation of vir genes in *Agrobacterium tumefaciens* (Bolten *et al.*, 1986; Machida *et al.*, 1986). Acetosyringone can therefore be used to increase *Agrobacterium*-mediated transformation frequencies *in vitro*. Shiekholeslam and Weeks, 1987 reported an increase from 2-3% in transformation frequency without acetosyringone to 55-63% with acetosyringone in *Arabidopsis*. In our initial experiments for both Oak and Sitka Spruce cultures no significant difference between acetosyringone concentrations was observed. However, later experiments revealed that acetosyringone did have a significant effect on the number of *GUS* expressing cells of both Oak and Sitka Spruce. Optimum concentrations for Oak were between 20 and 30 μM acetosyringone expressing a 5-fold increase in the number of *GUS* expressing loci transformed in a 50% bacterial dilution for 180min with a 72h co-cultivation period. Similar acetosyringone concentrations were observed for Sitka Spruce ESMs incubated for 60min in a 50% dilution of *Agrobacterium* with a 72h co-cultivation time. The number of *GUS* expressing cells increased 6.5-fold from 0-25 μM acetosyringone. While transforming hybrid poplar suspensions Howe *et al.*, 1994 cultured *Agrobacterium* overnight in 200 μM acetosyringone. Results on Oak and Sitka Spruce revealed that transformation efficiency decreased at higher acetosyingone concentrations. Mathews *et al.*, 1990 failed to transform *A. belladona* leaf explants in the absence of acetosyringone in the bacterial culture but obtained transgenic *A. belladona* plants resistant to kanamycin

after infecting the leaf explants with *Agrobacterium tumefaciens* cultured with 20 μM acetosyringone. The addition of acetosyringone to the bacterial culture media prior to transformation of Sitka Spruce ESMs (Drake *et al.*, 1997) was found to significantly increase transformation although significant differences in transformation frequency were not found with this compound at 50, 100, 150 μM acetosyringone. They did not examine the effect of preculture at acetosyringone concentrations below 50 μM and therefore transformation efficiencies at lower acetosyringone concentrations for Sitka Spruce ESMs on solid medium are unknown. James *et al.*, 1993 also reported that acetosyringone added to the induction medium for 5h prior to leaf disc inoculation significantly increased *GUS* gene expression and was only necessary during the induction phase and not during the co-cultivation period.

The effect of gene constructs especially the regulatory sequence, on transgene expression has been observed in coniferous tissue (Haggman *et al.*, 1998). In our studies the plant virus promoter CaMV 35S was used for all transformations. This CaMV 35S promoter and the NOS promoter have previously been shown to be active in conifers (Bekkaoui *et al.*, 1988; Dandekar *et al.*, 1987; Gupta *et al.*, 1988; Sederoff *et al.*, 1986). Increased levels of expression can however be achieved with the tandem repeat CaMV 35S (Bekkaoui *et al.*, 1990) or by using a double 35S promoter alone or coupled to translation enhancers (Charest *et al.*, 1993; Walters *et al.*, 1994). Reports suggest that other promoters such as ABA inducible promoters (Duchesne and Charest, 1992) may also increase expression. They reported that transient β-glucuronidase expression was higher in embryogenic cells of *Larix* X *eurolepis* and *Picea mariana* following micropropagation with the use of wheat abscisic acid inducible Em gene promoter. The Em promoter was inducible with abscisic acid and upon addition to the culture medium β-glucuronidase expression was increased 4-fold for *Picea mariana*.

Recent reports suggest that the use of introns could increase gene expression. The use of introns to increase heterologous gene expression could be important to achieve the required levels of genetic activity and to develop high level expression vectors for the transformation of conifers (Humara *et al.*, 1998). Intron enhancement of gene expression has been reported in various monocot species using certain introns but the situation in dicot species is less clear. In dicot species monocot introns do not stimulate and often reduce expression where effects of dicot introns vary from no stimulation of gene expression to a slight stimulatory effect (Vain *et al.*, 1996). Humara *et al.*, 1998 reported an increase in gene expression in pine species by varying the type of intron used.

The binary vector pBI121 was utilised in all transformations carried out in this thesis. However the production of transgenic plant lines is often complicated by the lack of unique restriction sites suitable for cloning into the large binary vectors used for *Agrobacterium*-mediated transformation. Hennegan *et al.*, 1998 have constructed a binary vector pBIN20 which contains a large multi cloning site (MCS) as well as a selectable kanamycin resistance gene *npt* II between the Ti border sequences. The progenitor plasmid for pBIN20 was pBI121.

4.4 Conclusion

Optimum *Agrobacterium* dilutions, incubation times and co-cultivation times were estimated for *Agrobacterium*-mediated transformation of Oak and Sitka Spruce embryogenic suspension cultures with *Agrobacterium* strain LBA4404::pBI121. Acetosyringone was proven to increase the number of *GUS* positive loci per gram of Oak and Sitka Spruce cells at approximately 25μM for both species. Stably transformed cultures of Oak and Sitka Spruce however, did not express the transferred genes for long enough to permit a molecular analysis of the transferred transgenes and therefore experimentation into choice of *Agrobacterium* strain and possibly the use of different promoters such as the ABA promoter need to be examined. Suggestions into the use of introns were discussed and perhaps future work could include these types of investigations to establish stable transformants.

Optimized *Agrobacterium*-mediated Transformation of Oak Embryogenic Suspension Cultures Based on Expression of *GUS* A gene.

LBA4404::pBI121 cultured in 100 cm^3 N.B. (50 $mgdm^{-3}$ kanamycin, 25μM acetosyringone) (filter sterilized)

↓ 30°C, 100rpm, overnight

7d old flasks of Oak embryogenic cultures sieved (100 μm mesh) and weighed

↓

50% *Agrobacterial* dilution prepared with filtrate (conditioned medium) in 100 cm^3 in 250 cm^3 sterile Erlenmeyer flask

↓

Cell samples (approx. 8 g) incubated in *LBA4404::pBI121* dilution

↓ 120 min, 100 rpm, growth room

Cells sieved (100 μm mesh) and washed twice in sterile dH_2O

↓

Washed cells immersed in 50cm^3 MS minus antibiotics and transferred to growth room

↓ co-cultivation 72h, 100 rpm

1 g samples removed for histochemical assay
500 $mgdm^{-3}$ cefotaxime added to remaining cells in flask (filter sterilized)

↓ 72h, 100 rpm, growth room

Cell sample (1 g) removed for histochemical assay
30 $mgdm^{-3}$ paromomycin (filter sterilized) added to remaining cells in flask

↓ 100rpm, growth room

Transformations subcultured as normal every 10d (MS, 500 $mgdm^{-3}$ cefotaxime, 30 $mgdm^{-3}$ paromomycin) Assays performed every 5d.

Optimized *Agrobacterium*-mediated Transformation of Sitka Spruce Embryonal Suspensor Masses Based on Expression of *GUS* A gene.

LBA4404::pBI121 cultured in 100 cm^3 N.B. (50 $mgdm^{-3}$ kanamycin, 25µM acetosyringone) (filter sterilized)

↓ 30°C, 100rpm, overnight

7d old flasks of sitka spruce embryonal suspensor masses sieved (100 µm mesh) and weighed

↓

50% *Agrobacterial* dilution prepared with filtrate (conditioned medium) in 100 cm^3 in 250 cm^3 sterile Erlenmeyer flask

↓

Cell samples (approx. 8 g) incubated in *LBA4404::pBI121* dilution

↓ 60 min, 100 rpm, growth room

20 cm^3 samples removed and placed in 25 cm^3 sterile plastic universal bottles and washed twice in sterile dH_2O

↓ 10 min, 3,500 g

Washed cells immersed in 50cm^3 sitka spruce embryogenesis, embryo initiation medium minus antibiotics and transferred to growth room

↓ co-cultivation 72h, 100 rpm

1 g samples removed for histochemical assay
500 $mgdm^{-3}$ cefotaxime added to remaining cells in flask (filter sterilized)

↓ 72h, 100 rpm, growth room

Cell sample (1 g) removed for histochemical assay
3 $mgdm^{-3}$ paromomycin (filter sterilized) added to remaining cells in flask

↓ 100rpm, growth room

Transformations subcultured as normal every 10d (Sitka Spruce embryogenesis, embryo initiation medium, 500 $mgdm^{-3}$ cefotaxime,3 $mgdm^{-3}$ paromomycin) Assays performed every 5d.

Chapter 5

Maturation and Regeneration of Oak (*Quercus robur* L.) and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Embryogenic Cultures

5.1 Introduction

Somatic embryogenesis provides the basis for genetic improvement of many forest species. These transgenic forest trees might be recovered from transformed embryogenic tissue. However, the low conversion frequency of somatic embryos into rooted plantlets often causes a bottleneck and remains a problem for the commercial utilization of this technology (Tautorus *et al.*, 1991).

Plant regeneration from Oak somatic embryos has been achieved for *Q. rubra* (Gingus and lineberger, 1989) and *Q. robur* (Chalupa, 1990) however at a low frequency. The absence (*Q. suber* (El Maataoui and Espagnac, 1987), *Q. ilex* (Feraud-Keller and Espagnac, 1989)) or low frequency (*Q. robur L., Q. suber*) conversion of somatic embryos into plantlets poses a serious problem for Oak regeneration. The development of somatic embryos is often blocked after the formation of cotyledons. The maturation and conversion of embryos involves a sequence of medium changes and the alteration of physical changes (Chalupa, 1995). *Quercus* is one of the forest tree species which is sensitive to added cytokinin. A variety of cytokinins have been used in attempts to regenerate Oak somatic embryos. Both benzylaminopurine and thidiazuron have been reported to stimulate shoot multiplication of some forest species (Chalupa, 1987). *Quercus robur* L. is one of the *Quercus* species which was found to react sensitively to added cytokinin even at a low frequency. Medium supplemented with a variety of auxins (GA_3, IBA, 2,4-D) were also beneficial in Oak regeneration.

The maturation and regeneration of Sitka Spruce to plantlets has been achieved (Krogstrup, 1988). Maturation of Sitka Spruce somatic embryos beyond stage 1 also requires changes in the initiation and maintenance medium (John *et al.*, 1995). Somatic embryos can develop into rooted plantlets on medium with reduced or eliminated

phytohormones. The use of abscisic acid in medium before transfer to hormone free medium for final germination was found to be highly beneficial (Tautorus *et al.*, 1991, Krogstrup *et al.*, 1988, Dunstan *et al.*, 1988).

In spite of successes, most woody plants still appear to be recalcitrant and regeneration of plants is rare. In order to regenerate transgenic plants from transformed embryogenic tissue it was imperative to formulate a successful combination of phytohormones in our media.

The following set of experiments outlines a number of medium combinations incorporating cytokinins and auxins in an attempt to regenerate rooted plantlets from somatic embryos of Oak and Sitka Spruce.

Results presented below show successful regeneration on devised medium combinations. Results recorded root and shoot proliferation as well as callus greening, cotyledon formation and the production of anthocyanin.

5.2 Results

5.2.1 Maturation and Regeneration of Oak (*Quercus robur* L.) Embryogenic Callus Experiment 1

Experiment 1 was based on the use of a desiccation treatment reported to be beneficial for the embryo conversion of *Q. petraea* somatic embryos (Chalupa, 1995) and *Q. robur* cultures (Ostrolucka *et al.*, 1995). The supplementation of maturation medium with abscisic acid was also investigated. Kim *et al.*, 1997 reported the successful germination of somatic embryo cultures of *Q. acutissima* in medium containing 0.1 $mgdm^{-3}$ ABA.

Half strength Murashige and Skoog medium was prepared as normal with the addition of 0%-12% sorbitol increasing in increments of 2%. To the autoclaved medium 0.2 $mgdm^{-3}$ BAP and 0 μM-100 μM ABA was added to each sorbitol concentration. For media containing 0% sorbitol 2 pieces of callus were added to each plate for each ABA concentration. Every 2d the calli were transferred to fresh dishes of the same medium but with increased levels of sorbitol *i.e.* 2%-12%. Control plates were set up which contained 0μM ABA and 0% sorbitol. Control plates remained untouched throughout the experiment. All cultures were grown in the dark in the growth room. When the 12% sorbitol level was reached approximately half of the cultures were transferred to plates containing ½ MS plus 1 $mgdm^{-3}$ BAP. The remaining half were transferred to ½ MS, 2% activated charcoal, 1 $mgdm^{-3}$ BAP. Cultures were grown in the light and transferred to fresh medium every 4-6 weeks.

Figure 5.1 shows root and shoot proliferation and anthocyanin production. Low rates of root and shoot development with 0% shoot formation for all ABA concentrations and only 15% root production (Plate 5.1) for 0 μM ABA were observed. Anthocyanin production for all ABA concentrations was evident and an indication that plants were possibly under some stress.

Figure 5.3 shows results on transfer of germinating embryos to ½ MS containing activated charcoal. The graph indicates zero root and shoot production for all ABA concentrations but continued embryo formation. Again, there was a relatively high level of anthocyanin production (Plate 5.2) for ABA concentrations but no anthocyanin production in the absence of ABA. Plate 5.3 shows the production of a number of cotyledonary abnormalities.

Results on ½ MS showed very low root and shoot development. In the presence and absence of activated charcoal there was no root or shoot formation at all ABA concentrations. However, at 0 μM ABA there was a low level of root production in the absence of activated charcoal. Therefore, results here suggest that ABA was of no benefit in proliferation of roots and shoots on Oak embryogenic callus.

5.2.2 Oak (*Quercus robur* L.) Embryogenic Callus Maturation and Regeneration Experiment 2

An alternative medium needed to be looked at for Oak embryogenic callus regeneration. We decided to look at Woody Plant Medium as that alternative. Chalupa, 1995 used WPM to some benefit in the maturation and regeneration of *Q. robur* embryogenic callus cultures.

This experiment was identical to experiment 1 above with the exception that instead of ½ MS we used WPM. Analogous concentrations of ABA and sorbitol were used and cultures treated in exactly the same manner.

Figure 5.2 shows results of transferring cultures onto WPM in the absence of sorbitol and ABA after desiccation treatment and exposure to ABA on WPM for 14d. Again results showed anthocyanin production however at a lower level than with ½ MS. There was an absence of shoot development but results for rooting were more favourable. Although root formation was only 10% for 0 μM, 1 μM and 10 μM ABA it was an improvement on the 0% recorded for ½ MS.

Figure 5.4 show results of callus regeneration on transfer to WPM plus 2% activated charcoal. Results showed that anthocyanin production was reduced compared to ½ MS. However, in contrast to results on WPM minus activated charcoal there was root formation on 0 μM ABA but not in the presence of 1 μM and 10 μM ABA. Again with the above experiments shoots failed to form.

Results on WPM were slightly improved compared to ½ MS for root and anthocyanin production but also failed to produce any shoots. A medium or medium

component that would promote shooting needed to be investigated. The aim of the following experiments was to find these components.

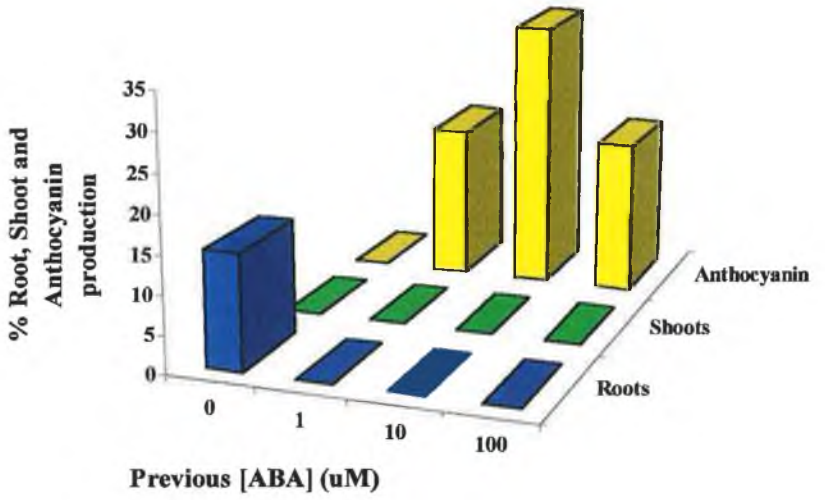


Figure 5.1
Formation of Oak roots, shoots and anthocyanin production on ½ MS medium plus 0 µM ABA, 0% sorbitol and 1 $mgdm^{-3}$ BAP following a 14d desiccation treatment with sorbitol and subjection to varying ABA concentrations on ½ MS. Percentages were calculated by scoring 10 calli per treatment. Experiments were carried out in duplicate.

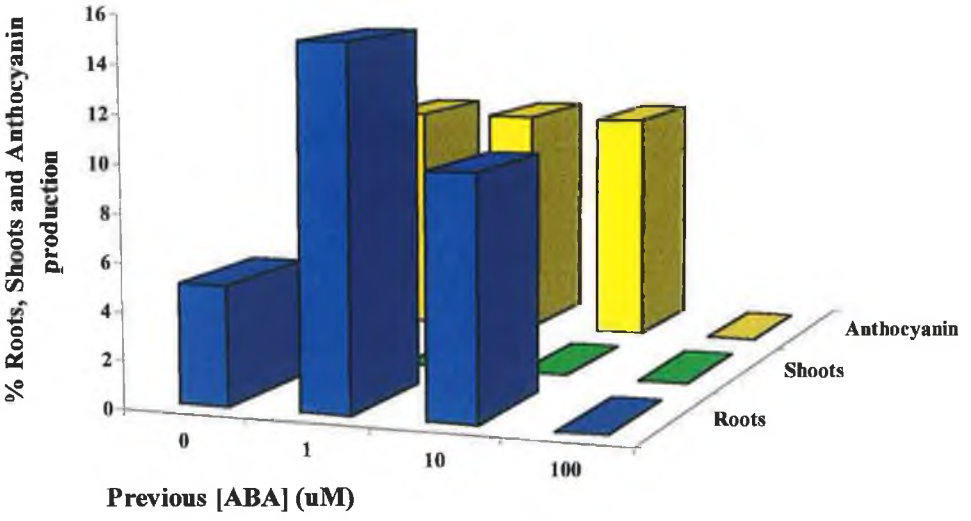


Figure 5.2
Figure shows formation of Oak roots, shoots and anthocyanin production on WPM with 0 µM ABA and 0% sorbitol following a 14d sorbitol desiccation treatment and subjection to varying concentrations of ABA on WPM. Percentages were calculated by scoring 10 calli per treatment. Experiment were carried out in duplicate.

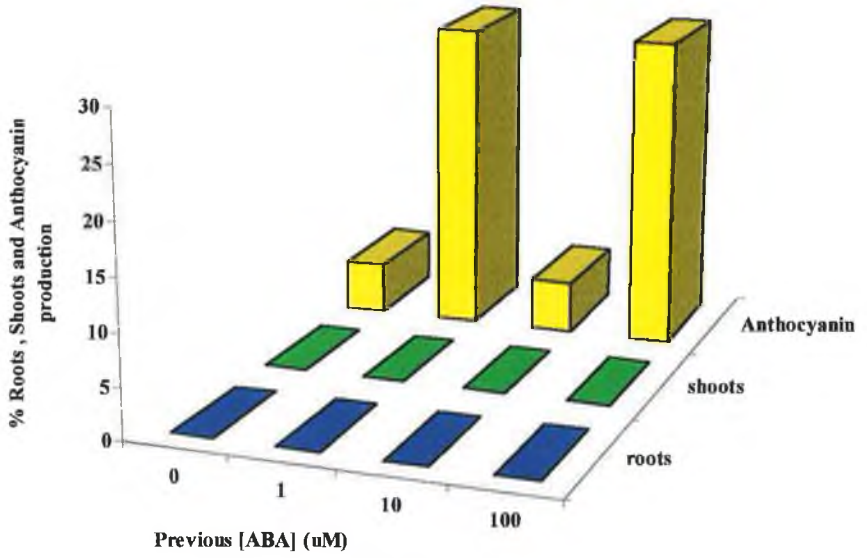


Figure 5.3
Regeneration of Oak embryogenic callus cultures. Figure shows the formation of roots, shoots and anthocyanin production on ½ MS plus 0 μM ABA, 0% sorbitol, 2% activated charcoal and 1mgdm^{-3} BAP. Cultures were transferred from ½ MS following a 14d desiccation treatment with sorbitol in the presence of varying ABA concentrations. Percentages were calculated by scoring 10 calli per treatment. Experiments were carried out in duplicate.

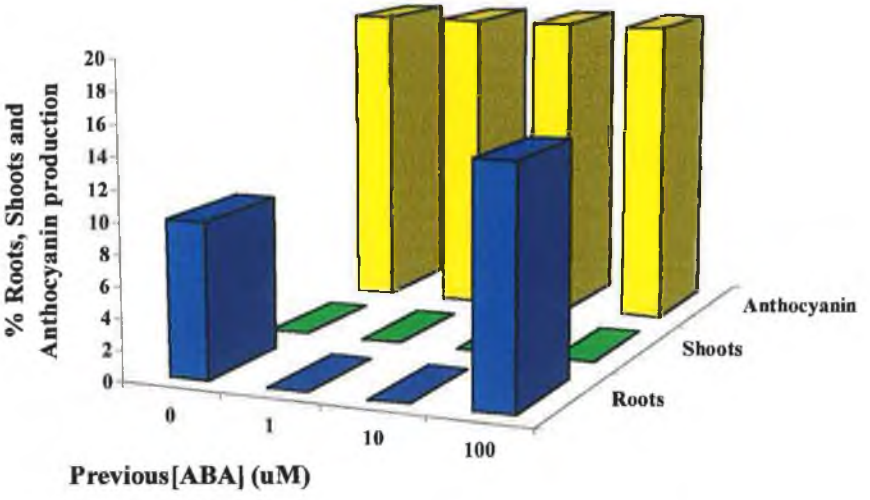


Figure 5.4
Regeneration of Oak from embryogenic callus cultures. Figure shows formation of roots, shoots and anthocyanin production on WPM with 0 μM ABA, 0% sorbitol, 2% activated charcoal and 1mgdm^{-3} BAP following a 14d desiccation treatment with sorbitol and subjection to varying concentration of ABA on WPM. A total of 10 calli were scored for each treatment and percentages recorded. Percentages were calculated by scoring 10 calli per treatment. Experiments were carried out in duplicate.

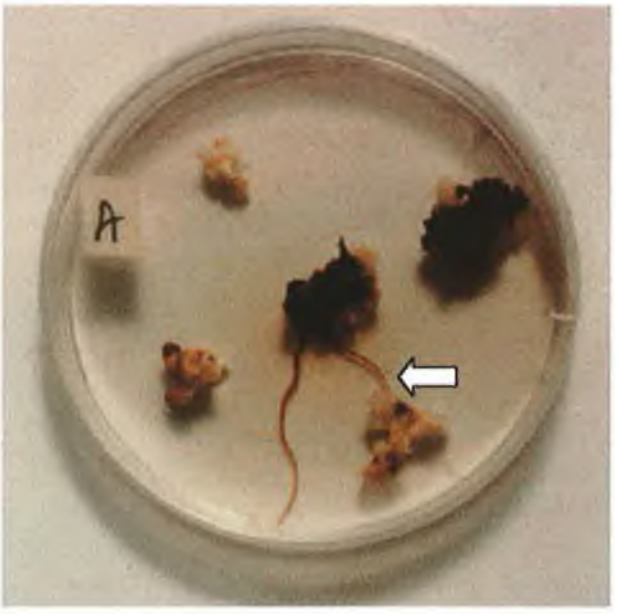


Plate 5.1

Formation of roots (arrow) on Oak embryogenic callus on ½ MS medium plus 0μM ABA, 0% sorbitol and 1 $mgdm^{-3}$ BAP. Cultures were transferred from ½ MS plus 12% sorbitol desiccation treatment in the absence of ABA. 9 cm Petri dishes.

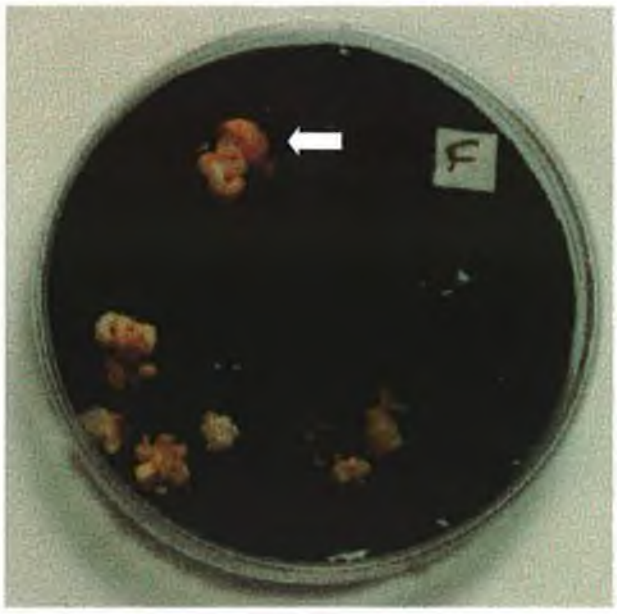


Plate 5.2

Maturation of Oak embryogenic callus on ½ MS medium plus 2% activated charcoal and 1 $mgdm^{-3}$ BAP. Cultures were transferred from ½ MS with a 12% sorbitol desiccation treatment plus 100 μM ABA. Plate shows the absence of roots and shoots but the production of large amounts of anthocyanin (arrow). 9 cm Petri dishes.

Plate 5.3

Maturation of oak embryogenic callus on ½ MS plus 2% activated charcoal and 1 $mgdm^{-3}$ BAP. Cultures had been transferred from ½ MS plus 12% desiccation treatment in the presence of 10 μM ABA. The plate shows the absence of roots, shoots and anthocyanin production. A number of cotyledonary abnormalities can be observed (arrows). 9 cm Petri dishes.

5.2.3 Oak (*Quercus robur* L.) Embryogenic Callus Maturation and Regeneration Experiment 3

Chalupa, 1995 reported the benefits of IBA, 2,4-D and GA^3 in the regeneration of Oak (*Quercus robur* L.) embryogenic callus cultures. A number of medium components were investigated in this set of experiments.

Murashige and Skoog medium and Woody Plant Medium were prepared as normal. Concentrations of 0.5 $mgdm^{-3}$, 1 $mgdm^{-3}$ and 2 $mgdm^{-3}$ BAP were added to the autoclaved medium either alone or in combination with 0.5 $mgdm^{-3}$ or 1 $mgdm^{-3}$ 2,4-D or 0.1 $mgdm^{-3}$ or 1 $mgdm^{-3}$ IBA or 0.5 $mgdm^{-3}$ or 1 $mgdm^{-3}$ GA_3. Ten plates were set aside for each combination and 5 pieces of callus were cultured per plate and transferred to the growth room in the dark for 6–8 weeks. One third of each combination was then transferred to WPM plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$ or 0.6 $mgdm^{-3}$ BAP and grown in the light or the dark. Cultures were subcultured every 4–6 weeks onto fresh medium. All cultures were scored for greening and cotyledon formation. All medium combinations were carried out in duplicate.

Results on WPM showed high callus greening on transfer of cultures from WPM plus 0.5 $mgdm^{-3}$, 1 $mgdm^{-3}$ and 2 $mgdm^{-3}$ BAP in the absence (Fig. 5.5) and presence of 0.1 $mgdm^{-3}$ IBA (Fig. 5.6) and 1 $mgdm^{-3}$ IBA (Fig. 5.7) to 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$ and 0.6 $mgdm^{-3}$ BAP on WPM in the light. Cotyledon formation was recorded at a high level for all combinations. However, on transfer to WPM combinations and culture in the dark results were significantly different. Callus greening was absent for all combinations (Fig. 5.8, 5.9, 5.10). Callus greening for all combinations was absent and cotyledon formation was reduced and recorded at a lower percentage.

Results recorded for the above combinations on ½ MS on transfer of cultures to growth in the light showed a reduction in both callus greening and cotyledon formation (Fig. 5.11) for all combinations with the exception of cultures transferred from 2 $mgdm^{-3}$ BAP, 1 $mgdm^{-3}$ IBA to 0.6 $mgdm^{-3}$ BAP where callus greening was recorded as 100% (Fig. 5.12).

Regeneration of cultures on ½ MS plus 0.5 $mgdm^{-3}$, 1 $mgdm^{-3}$ and 2 $mgdm^{-3}$ BAP in combination with 0.5 $mgdm^{-3}$ and 1 $mgdm^{-3}$ GA_3 and transfer to WPM plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$ and 0.6 $mgdm^{-3}$ BAP in the light showed a reduction in callus greening to 0% and a reduction in cotyledon formation (Fig. 5.13). Cotyledon formation was completely inhibited for cultures exposed to 0.5 $mgdm^{-3}$ GA_3 (Fig. 5.13) and 1 $mgdm^{-3}$ GA_3 (Fig. 5.14).

Exposure of cultures to BAP combinations plus 0.5 $mgdm^{-3}$ 2,4-D and 1 $mgdm^{-3}$ 2,4-D on ½ MS resulted in complete absence of callus greening (Fig. 5.16). Cotyledon formation was recorded at 100% for a number of combinations but was reduced however to 0% for cultures exposed to 1 $mgdm^{-3}$ BAP, 0.5 $mgdm^{-3}$ 2,4-D and 2 $mgdm^{-3}$ BAP, 0.5 $mgdm^{-3}$ 2,4-D to 0.2 $mgdm^{-3}$ BAP (Fig. 5.15).

For all medium combinations outlined above there was an absence of root and shoot proliferation.

Results for this set of experiments proved very unsatisfactory. Roots and shoots are absent for all medium combinations used. Callus greening and cotyledon formation was recorded at high levels on WPM medium for all combinations. Transfer of callus to culture in the dark resulted in complete inhibition of callus greening and a reduction in cotyledon formation on WPM medium. Attempted regeneration of Oak callus on ½ MS resulted in inhibition of greening for all combinations cultured in the light. Cotyledon formation slightly reduced but not significantly compared to callus cultured on

WPM. Results here correlate with results recorded in experiment 1 in that WPM appears to be more beneficial in the regeneration of Oak callus compared to ½ MS.

In conclusion it was evident that external auxins and cytokinins added to the two medium types did not have any significant effect on Oak regeneration rather it was the medium type used and whether cultures were grown in the light of dark which significantly affected regeneration.

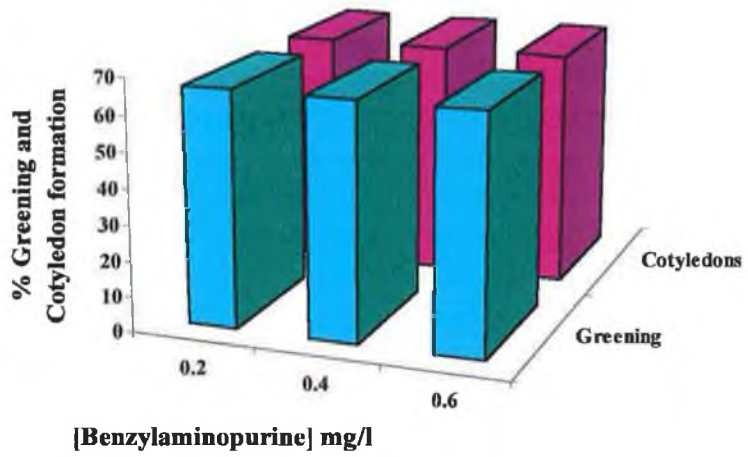


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Figure 5.5

Maturation of oak embryogenic callus cultures. Embryogenic callus had previously been cultured on WPM plus 0.5 $mgdm^{-3}$ (A), 1 $mgdm^{-3}$ (B) and 2 $mgdm^{-3}$ (C) benzylaminopurine for 4-6 weeks in the dark. Graphs A, B and C show % callus greening and cotyledon formation on transfer to WPM plus 0.2, 0.4 and 0.6 $mgdm^{-3}$ BAP in the light. Results were recorded from 3 plates each containing 5 callus pieces for each BAP concentration. Experiments were carried out in duplicate.

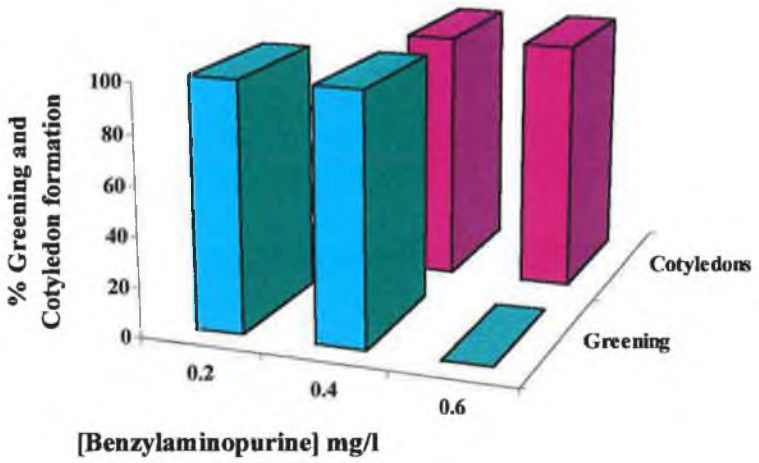

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Figure 5.6

Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on WPM plus 0.5 $mgdm^{-3}$ BAP, 0.1 $mgdm^{-3}$ IBA (A), 1 $mgdm^{-3}$ BAP, 0.1 $mgdm^{-3}$ IBA (B) and 2 $mgdm^{-3}$ BAP, 0.1 $mgdm^{-3}$ IBA (C) for 4-6 weeks in the dark. Graphs A,B and C show callus greening and cotyledon formation on transfer to WPM plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$, and 0.6 $mgdm^{-3}$ benzylaminopurine in the light. Results were recorded from 3 plates each containing 5 pieces of callus for each benzylaminopurine concentration. Experiments were carried out in duplicate.

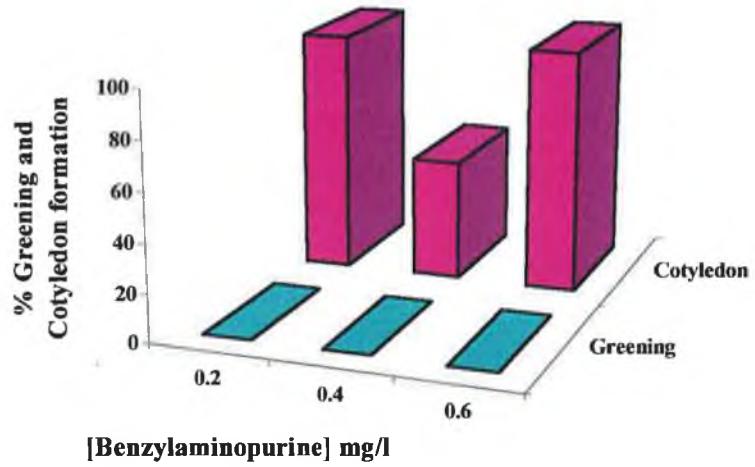

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Figure 5.7

Maturation of oak embryogenic callus cultures. Embryogenic callus had precviously been cultured on Woody Plant Medium plus 0.5 $mgdm^{-3}$ (A), 1 $mgdm^{-3}$ (B) and 2 $mgdm^{-3}$ (C) benzylaminopurine for 4-6 weeks in the dark. Graphs A, B and C show % callus greening and cotyledon formation on transfer to Woody Plant Medium plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$, and 0.6 $mgdm^{-3}$ benzylaminopurine in the dark. Results were recorded from 3 plates each containing 5 callus pieces for each benzylaminopurine concentration. Experiments were carried out in duplicate.

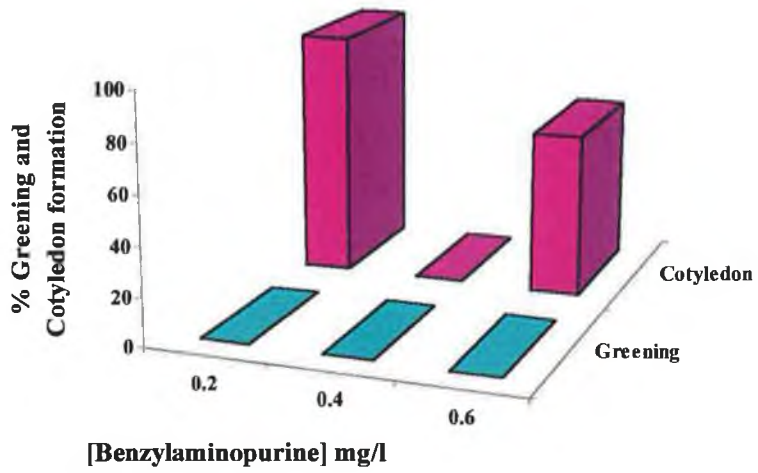

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Figure 5.8

Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on WPM plus 0.5 mgdm^{-3} BAP, 0.1 mgdm^{-3} IBA (A), 1 mgdm^{-3} BAP, 0.1 mgdm^{-3} IBA (B) and 2 mgdm^{-3} BAP, 0.1 mgdm^{-3} IBA (C) for 4-6 weeks in the dark. Graphs A, B and C show callus greening and cotyledon formation on transfer to WPM plus 0.2 mgdm^{-3}, 0.4 mgdm^{-3}, and 0.6 mgdm^{-3} BAP in the dark. Results were recorded from 3 plates each containing 5 pieces of callus for each BAP concentration. Experiments were carried out in duplicate.

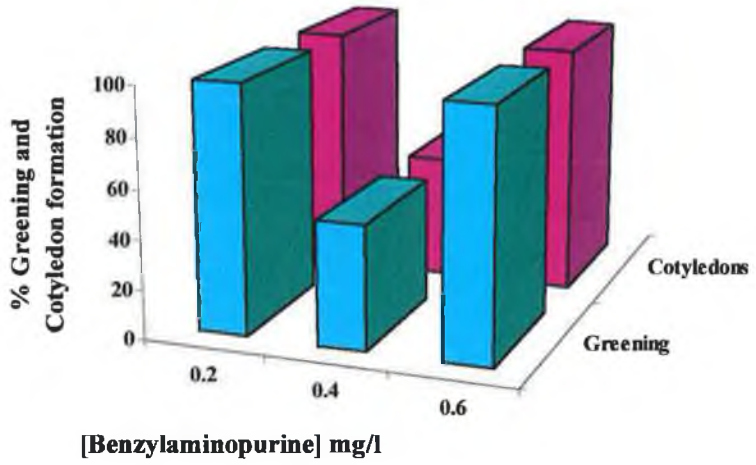

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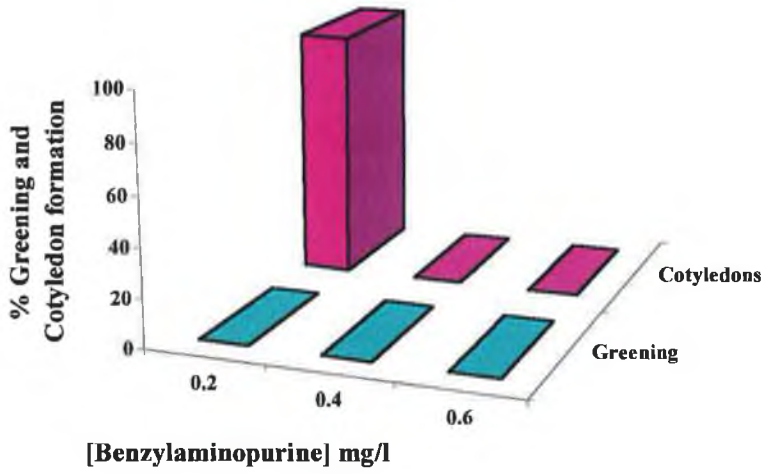
Figure 5.9

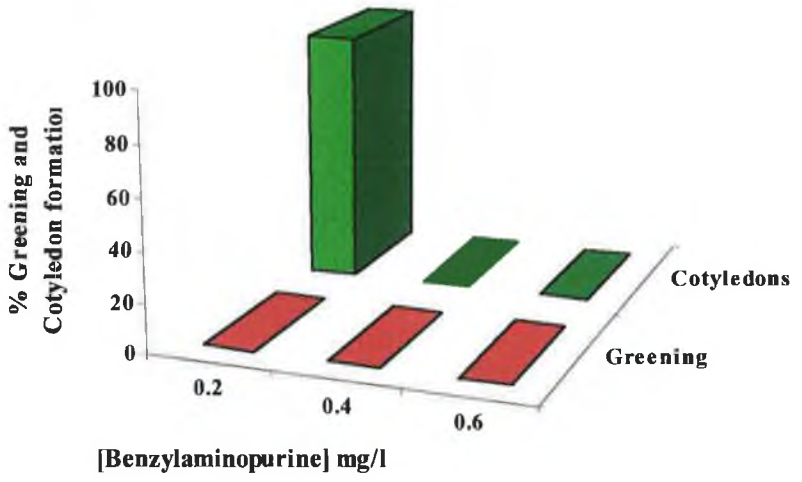
Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on WPM plus 0.5 $mgdm^{-3}$ BAP, 1 $mgdm^{-3}$ IBA (A), 1 $mgdm^{-3}$ BAP, 1 $mgdm^{-3}$ IBA (B) and 2 $mgdm^{-3}$ BAP, 1 $mgdm^{-3}$ IBA (C) for 4-6 weeks in the dark. Graphs A, B and C show callus greening and cotyledon formation on transfer to WPM plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$, and 0.6 $mgdm^{-3}$ BAP in the light. Results were recorded from 3 plates each containing 5 pieces of callus for each benzylaminopurine concentration. Experiments were carried out in duplicate.



C

Figure 5.10

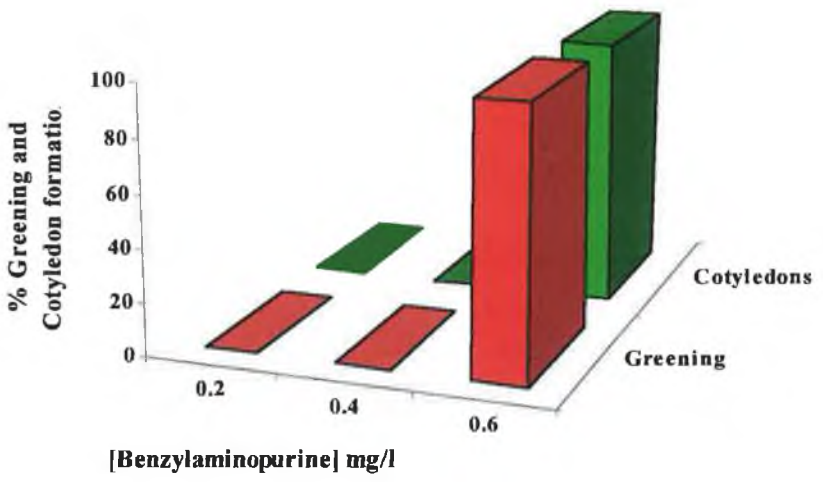
Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on WPM plus 0.5 mgdm^{-3} BAP, 1 mgdm^{-3} IBA (A), 1 mgdm^{-3} BAP, 1 mgdm^{-3} IBA (B) and 2 mgdm^{-3} BAP, 1 mgdm^{-3} IBA (C) for 4-6 weeks in the dark. Graphs A, B and C show callus greening and cotyledon formation on transfer to WPMplus 0.2 mgdm^{-3}, 0.4 mgdm^{-3}, and 0.6 mgdm^{-3} BAP in the dark. Results were recorded from 3 plates each containing 5 pieces of callus for each BAP concentration. Experiments were carried out in duplicate.



C

Figure 5.11

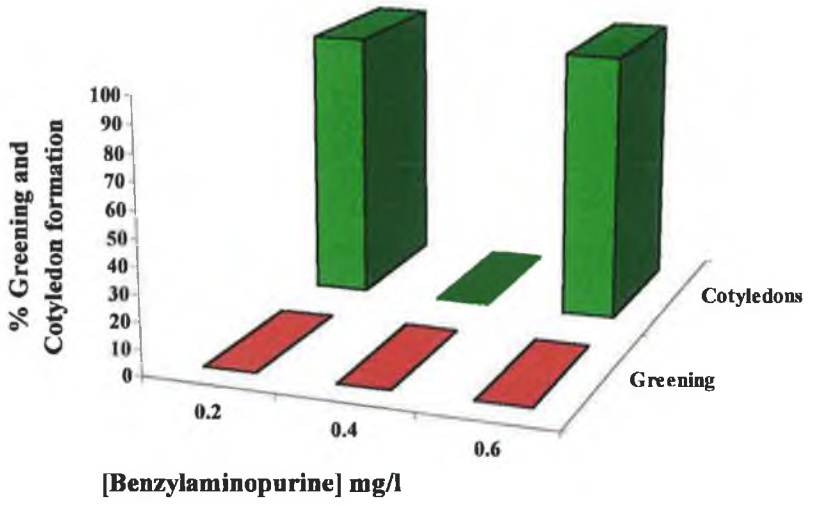
Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on ½ MS medium plus 0.5 $mgdm^{-3}$ BAP, 0.1 $mgdm^{-3}$ IBA (A), 1 $mgdm^{-3}$ BAP, 0.1 $mgdm^{-3}$ IBA (B) and 2 $mgdm^{-3}$ BAP, , 0.1 $mgdm^{-3}$ IBA (C) in the dark for 4-6 weeks. Graphs A, B and C show % callus greening and cotyledon formation on transfer to WPM plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$ and 0.6 $mgdm^{-3}$ BAP in the light. Results were recorded from 3 plates each containing 5 callus pieces for each BAP concentration. Experiments were carried out in duplicate.



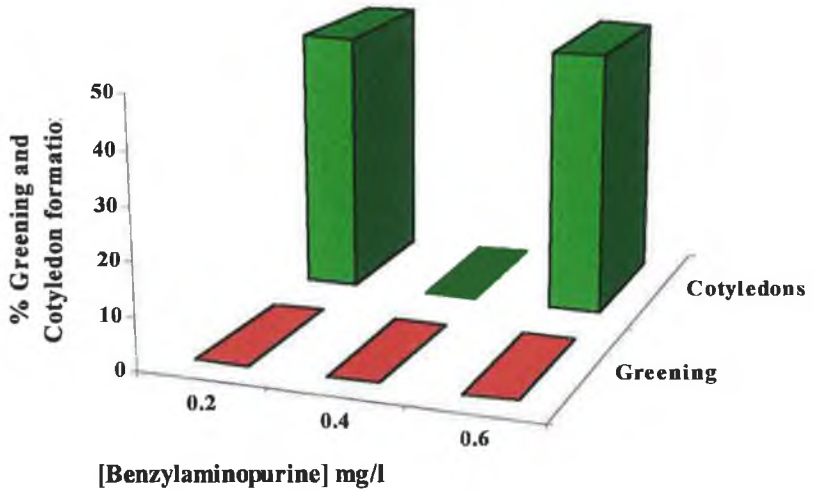
C

Figure 5.12

Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on ½ MS medium plus 0.5 $mgdm^{-3}$ BAP, 1 $mgdm^{-3}$ IBA (A), 1 $mgdm^{-3}$ BAP, 1 $mgdm^{-3}$ IBA (B) and 2 $mgdm^{-3}$ BAP, 1 $mgdm^{-3}$ IBA (C) in the dark for 4-6 weeks. Graphs A, B and C show % callus greening and cotyledon formation on transfer to WPM plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$ and 0.6 $mgdm^{-3}$ BAP in the light. Results were recorded from 3 plates each containing 5 callus pieces for each BAP concentration. Experiments were carried out in duplicate.



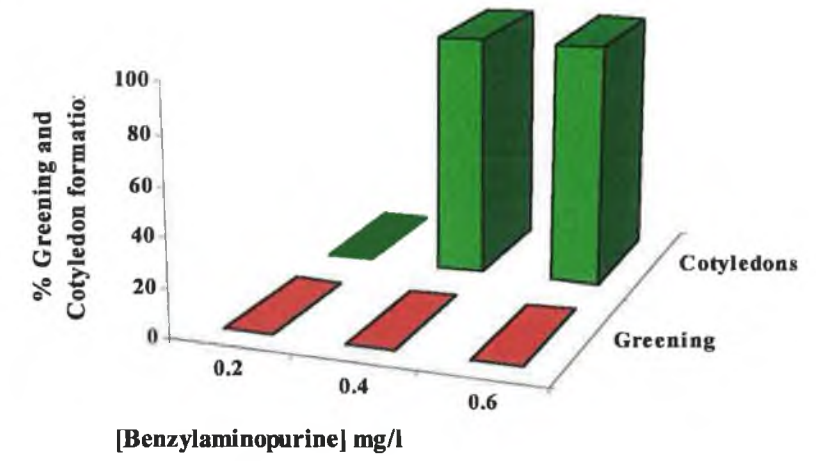
A



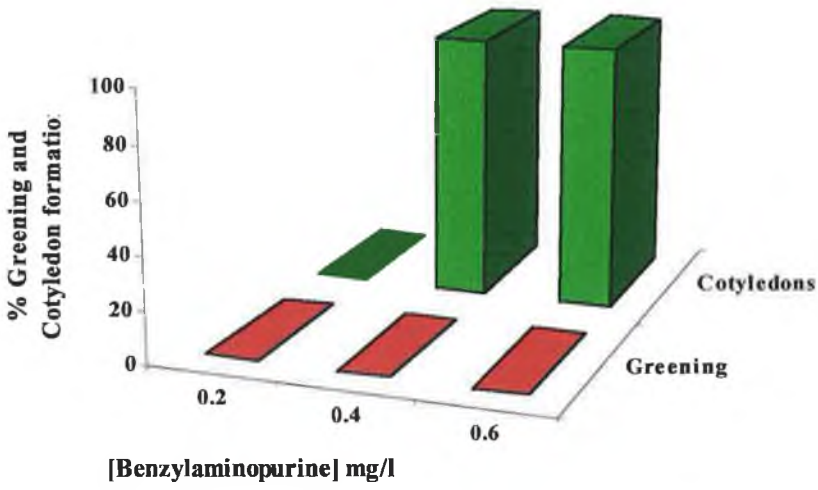
B

Figure 5.13

Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on ½ MS medium plus 0.5 mgdm^{-3} BAP, 0.5 mgdm^{-3} GA_3 (A) and 2 mgdm^{-3} BAP, 0.5 mgdm^{-3} GA_3 (B) in the dark for 4-6 weeks. Graphs A and B show % callus greening and cotyledon formation on transfer to WPM plus 0.2 mgdm^{-3}, 0.4 mgdm^{-3} and 0.6 mgdm^{-3} BAP in the light. Results for 1 mgdm^{-3} BAP, 0.5 mgdm^{-3} GA_3 there was no callus greening or cotyledon formation and therefore the results were not graphed. Results were recorded from 3 plates each containing 5 callus pieces for each BAP concentration. Experiments were carried out in duplicate.



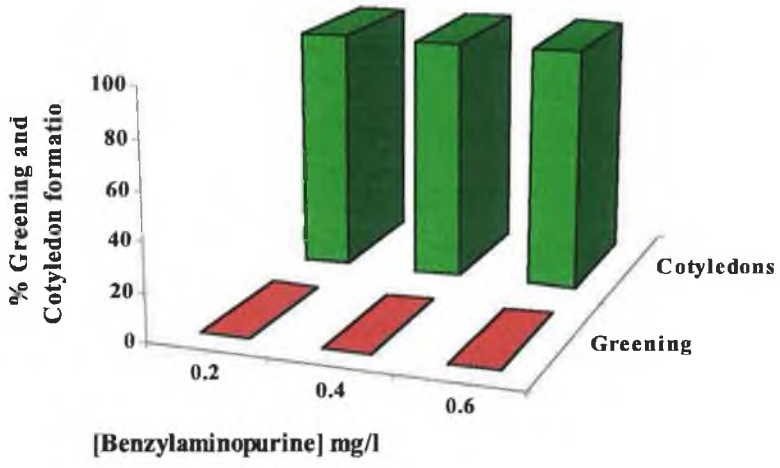
A



B

Figure 5.14

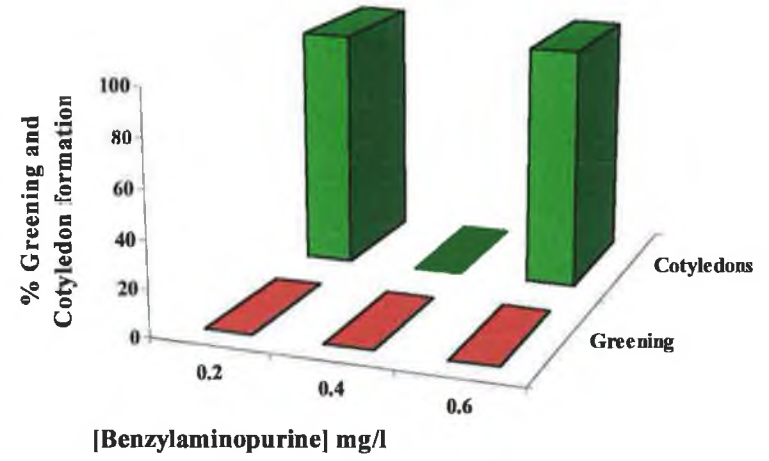
Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on ½ MS medium plus 0.5 mgdm^{-3} BAP, 1.0 mgdm^{-3} GA_3 (A), 1 mgdm^{-3} BAP, 1.0 mgdm^{-3} GA_3 (B). in the dark for 4-6 weeks. Graphs A and B show % callus greening and cotyledon formation on transfer to WPM plus 0.2 mgdm^{-3}, 0.4 mgdm^{-3} and 0.6 mgdm^{-3} BAP in the light. Results for 2 mgdm^{-3} BAP, 1.0 mgdm^{-3} GA_3 did not yield any cotyledons or callus greening and were not graphed. Results were recorded from 3 plates each containing 5 callus pieces for each benzylaminopurine concentration. Experiments were carried out in duplicate.



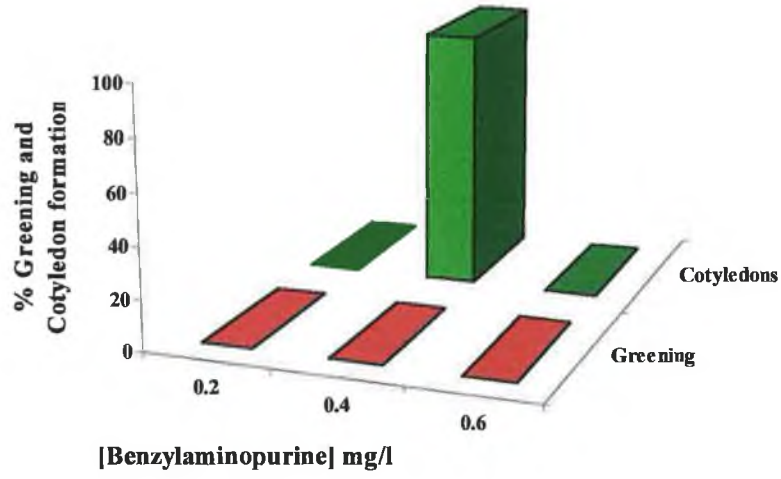
C

Figure 5.15

Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on ½ MS medium plus 0.5 $mgdm^{-3}$, 0.5 $mgdm^{-3}$ 2,4 D (A), 1 $mgdm^{-3}$ BAP, 0.5 $mgdm^{-3}$ 2,4 D (B) and 2 $mgdm^{-3}$ BAP, 0.5 $mgdm^{-3}$ 2,4 D in the dark for 4-6 weeks. Graphs A, B and C show % callus greening and cotyledon formation on transfer to WPM plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$ and 0.6 $mgdm^{-3}$ BAP in the light. Results were recorded from 3 plates each containing 5 callus pieces for each BAP concentration. Experiments were carried out in duplicate.



A



B

Figure 5.16

Maturation of oak embryogenic callus. Embryogenic callus had previously been cultured on ½ MS medium plus 0.5 $mgdm^{-3}$, 1.0 $mgdm^{-3}$ 2,4 D (A) and 2 $mgdm^{-3}$ BAP, 1.0 $mgdm^{-3}$ 2,4 D (B) in the dark for 4-6 weeks. Graphs A and B show % callus greening and cotyledon formation on transfer to WPM plus 0.2 $mgdm^{-3}$, 0.4 $mgdm^{-3}$ and 0.6 $mgdm^{-3}$ BAP in the light. Results were recorded from 3 plates each containing 5 callus pieces for each benzylaminopurine concentration. Experiments were carried out in duplicate.

5.2.4 Oak (*Quercus robur* L.) Embryogenic Callus Maturation and Regeneration Experiment 4

The following experiment was based on the use of thidiazuron. Thidiazuron has been described as being amongst the most active cytokinin–like substances for woody plant tissue culture. There are a number of reports on the effect of thidiazuron on shoot proliferation (Kim *et al.*, 1997; Bhagwat *et al.*, 1996)

Murashige and Skoog medium was prepared as normal. To the medium 0.3 $mgdm^{-3}$ gelrite was added. Concentrations of 0.5 μM and 1 μM TDZ were added to the autoclaved medium. For each TDZ concentration 5 plates were cultured each with 5 pieces of Oak embryogenic callus. Plates were transferred to the growth room and cultured in the dark for 3 d then exposed to the light for a further 3 weeks. Cultures exhibiting shoot formation should have been transferred to MS medium plus 2 μM NAA or 2 μM BAP and cultured in the light for root establishment.

All Oak embryogenic callus cultures before transfer to root establishment medium became extremely soft and did not exhibit any of the characteristics which regenerating Oak should exhibit. These characteristics include greening, cotyledon formation and root or shoot development. The experiment was abandoned before transfer to rooting medium as it was evident that root or shoot establishment would not occur.

5.2.5 Oak (*Quercus robur* L.) Embryogenic Callus Maturation and Regeneration Experiment 5

These experiments examined the effect of P24 medium (Eva Wilhelm and Renate Rodler, per comm) on shooting of Oak embryogenic callus cultures.

P24 medium was prepared as normal. Into one batch 0.9 μM BAP was added to the autoclaved medium. The second batch remained hormone free. Embryogenic cultures were plated onto the two medium types and left for 6 weeks in the growth room with half of the cultures subjected to the light and half grown in the dark. Cultures were then transferred to 6 types of maturation medium (Table 2.9) all of which were variations of P24 medium. For each maturation treatment we plated a total of 10 plates each with 5 pieces of callus from P24 medium plus 0.9 μM BAP and P24 medium minus BAP. Half of the plates were grown in the light and half in the dark. All cultures were subcultured every 5-6 weeks onto fresh medium. All experiments were carried out in duplicate.

Results showed a very high level of shoot proliferation on medium 1 and medium 3 (Fig. 5.17). However results for rooting proved less favourable with roots only occurring in medium 2 at 20%. Anthocyanin production was much reduced at 0% in most cases. Cultures transferred from P24 minus antibiotics from the dark to all maturation mediums in the light did not yield any roots, shoots or anthocyanin production.

Results of transferring cultures from P24 plus 0.9 μM BAP in the light to maturation media 2, 3, 4, 5 and 6 grown in the dark are outlined in Figure 5.18. Results

showed a high level of shoot proliferation. However embryos exhibiting both root and shoot development were recorded at a low percentage.

Figure 5.19 shows results on transfer of cultures from P24 medium minus hormones in the light to maturation media 3 and 5 in the dark. Again, results showed high levels of shoot formation, as high as 80% on medium 3. Root formation however, was low at 0% for medium 3 and 30% for medium 5.

Figure 5.20 shows results recorded on transfer of cultures from P24 medium plus 0.9 μM BAP in the light to maturation media 2, 3 and 6 in the light. Results showed high percentage root formation for medium 3 and 6 but no root formation for either medium type. Results from the transfer of cultures from P24 medium minus hormones in the light to all six maturation mediums in the light did not yield any roots, shoots or production of anthocyanin on cultures.

Figure 5.21 shows results on transfer of cultures from P24 medium plus 0.9 μM BAP in the dark to maturation media 2, 3, 4, 5 and 6 in the dark. A reduction in shoot development was recorded at 10% for medium 6 but 0% for all other media. Again root formation was recorded at very low levels. Anthocyanin production was present at low levels. Results recorded for transfer of cultures from P24 minus hormones in the dark to all six maturation media in the dark showed the absence of rooting, shooting and production of anthocyanin.

Results from experiments on P24 medium concluded that there was an increase in shoot proliferation and root development either alone or on the same embryo. A significant reduction in anthocyanin production was recorded. Although rooting increased it was at a low level and rarely occurred in collaboration with shooting. The

reduction in anthocyanin production indicated that embryogenic callus were not as stressed as previously in the presence of ABA. A medium has been devised that yields an increase in shooting and reduces anthocyanin production but it was not yielding enough of an increase in root proliferation from germinating embryos. It also appeared that a period of exposure to the light was favourable along with culture in the dark to promote this increase in shooting.

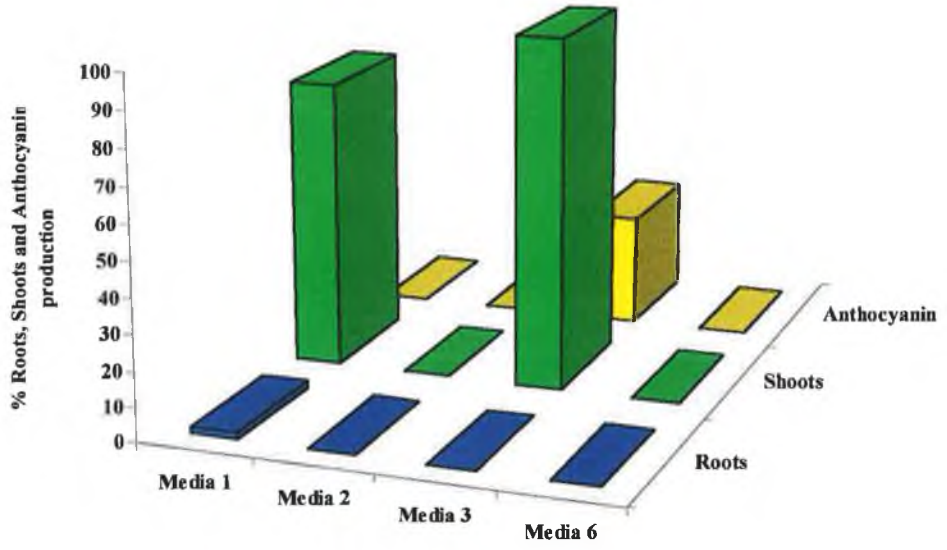


Figure 5.17

Regeneration of Oak embryogenic callus on P24 maturation medium. Formation of roots, shoots and the production of anthocyanin on P24 maturation media 1, 2, 3 and 6 in the light following 6 weeks growth on P24 medium plus 0.9 μM BAP in the dark. Maturation media not graphed did not exhibit any root, shoot or anthocyanin production. Percentages were calculated from 10 plates each containing 5 callus pieces per treatment. Results for P24 medium minus BAP did not show root, shoot or anthocyanin production for all 6 maturation media. Experiments were carried out in duplicate.

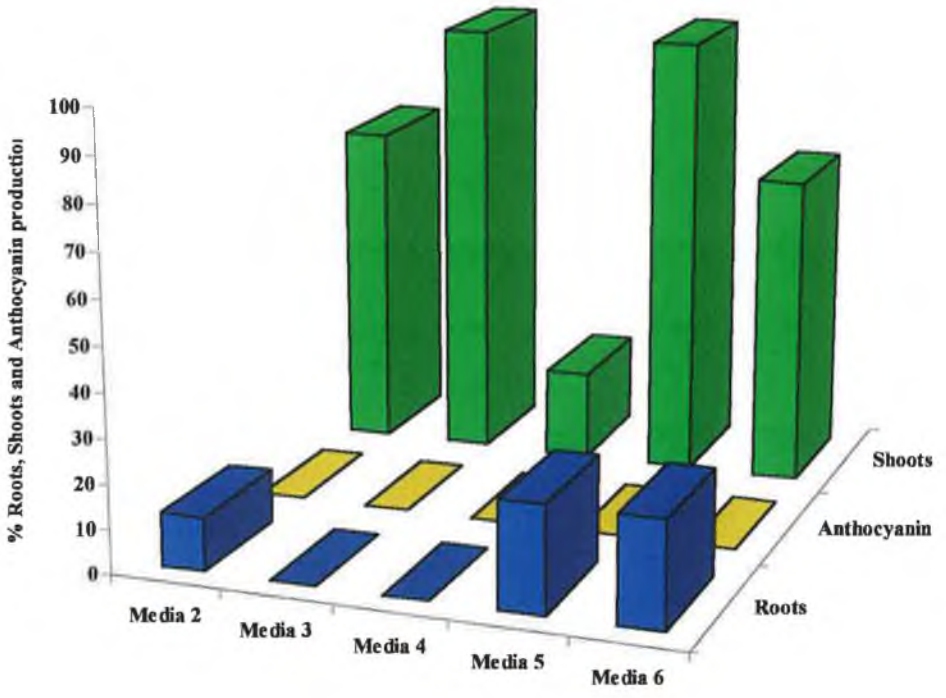


Figure 5.18

Regeneration of Oak embryogenic callus on P24 maturation medium. Formation of roots, shoots and production of anthocyanin on P24 maturation media 2, 3, 4, 5 and 6 in the dark following 6 weeks growth on P24 medium plus 0.9 μM BAP in the light. Maturation media not graphed failed to exhibit root, shoot or anthocyanin production. Percentages were calculated from 10 plates each containing 5 callus pieces per treatment. Experiments were carried out in duplicate.

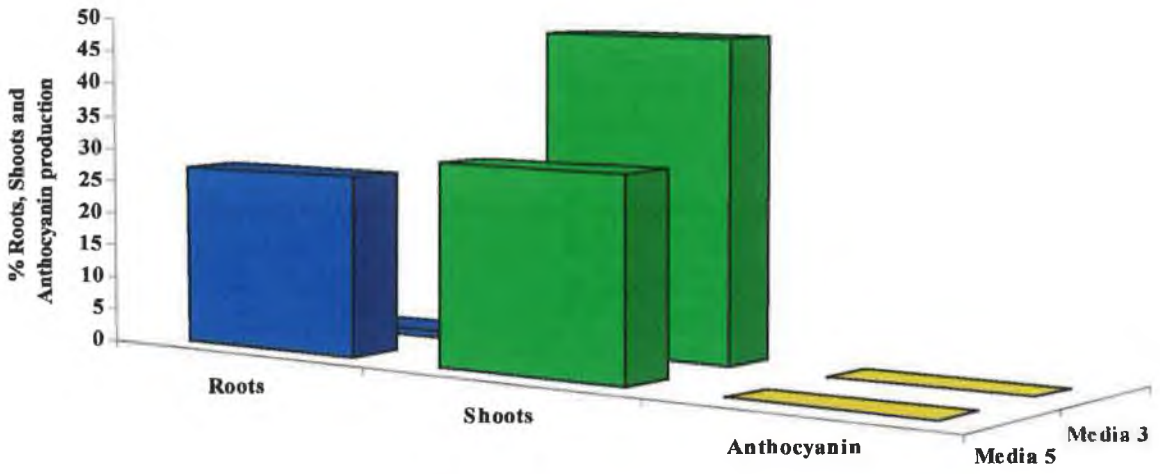


Figure 5.19

Regeneration of Oak embryogenic callus on P24 maturation medium. Formation of roots, shoots and the production of anthocyanin on P24 maturation media 3 and 5 in the dark following 6 weeks growth on P24 medium minus BAP in the light. Maturation media not graphed did not exhibit any root, shoot or anthocyanin production. Percentages were calculated from 10 plates each containing 5 pieces of callus per treatment. Experiments were carried out in duplicate.

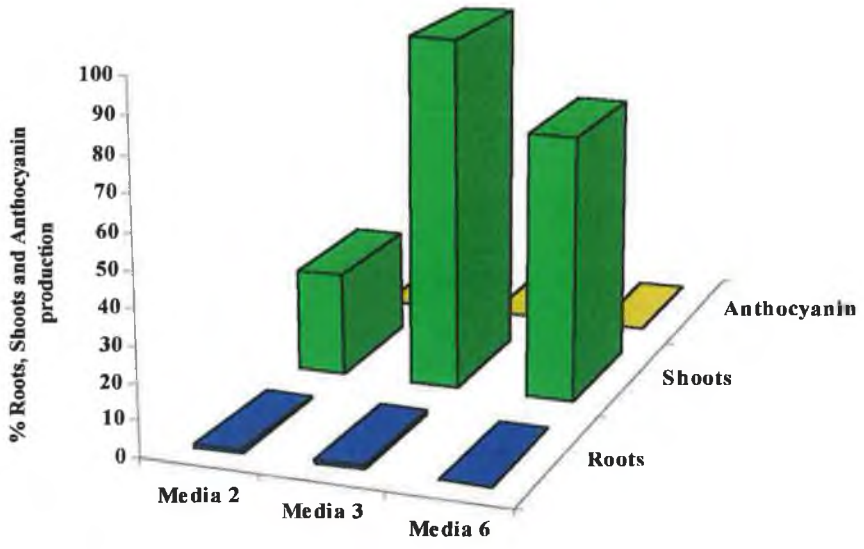


Figure 5.20

Regeneration of Oak embryogenic callus on P24 maturation medium. Formation of roots, shoots and the production of anthocyanin on P24 maturation media 2, 3 and 6 in the light following 6 weeks growth on P24 medium plus 0.9 μM BAP in the light. Maturation medium not graphed did not exhibit any root, shoot or anthocyanin production. Percentages were calculated from 10 plates each containing 5 calli pieces per treatment. Results for P24 medium minus BAP showed no root, shoot or anthocyanin production and results were therefore not presented. Experiments were carried out in duplicate.

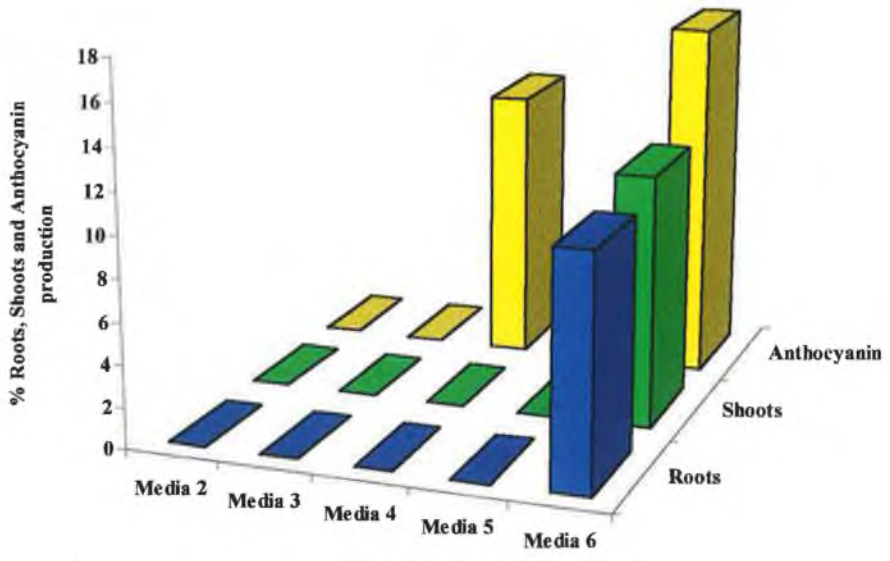


Figure 5.21

Regeneration of Oak embryogenic callus on P24 maturation medium. Formation of roots and shoots and the production of anthocyanin on P24 maturation media 2, 3, 4, 5 and 6 in the dark following 6 weeks growth on P24 medium plus 0.9 μM BAP in the dark. Maturation media not graphed did not exhibit roots, shoots or anthocyanin production. Percentages were calculated from 10 plates each containing 5 callus pieces per treatment. Results for P24 medium minus BAP showed no root, shoot or anthocyanin production and results were therefore not graphed. Experiments were carried out in duplicate.

5.2.6 Oak (*Quercus robur* L.) Embryogenic Callus Maturation and Regeneration Experiment 6

Following results from the previous experiment it appeared that this P24 medium plus 0.9 μM BAP was proving to be the most beneficial for Oak embryogenic callus regeneration. P24 maturation media 1 and 6 exhibited the best results. On observation of Oak callus on these plates it became evident that although not recorded in graphs as a number became contaminated, P24 medium 1 yielded callus which looked most like maturing into full plantlets complete with a root and shoot.

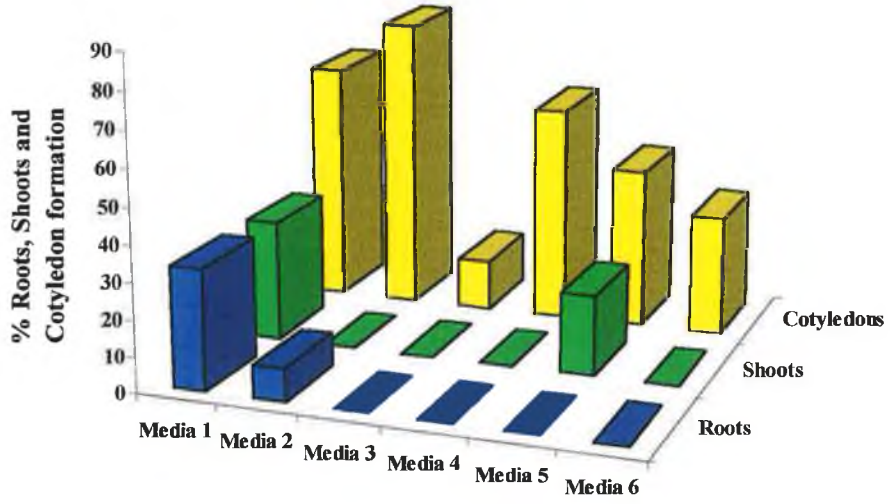
P24 medium was prepared as normal and into the autoclaved medium 0.9 μM BAP was added. Oak embryogenic callus was cultured on 30 plates each containing 5 pieces of callus. Cultures were transferred to the growth room and cultured in the light for 5–7 weeks. The six P24 maturation media were prepared and for each medium 5 plates were allocated from the 0.9 μM BAP P24 medium. Cultures were transferred to the growth room and half of the plates cultured in the light and half cultured in the dark for a further 7 weeks.

All cultures were then transferred to P24 medium 1 and subcultured onto fresh P24 medium 1 every 5 weeks. Each piece of Oak callus was scored for roots, shoots and cotyledon formation at each subculture.

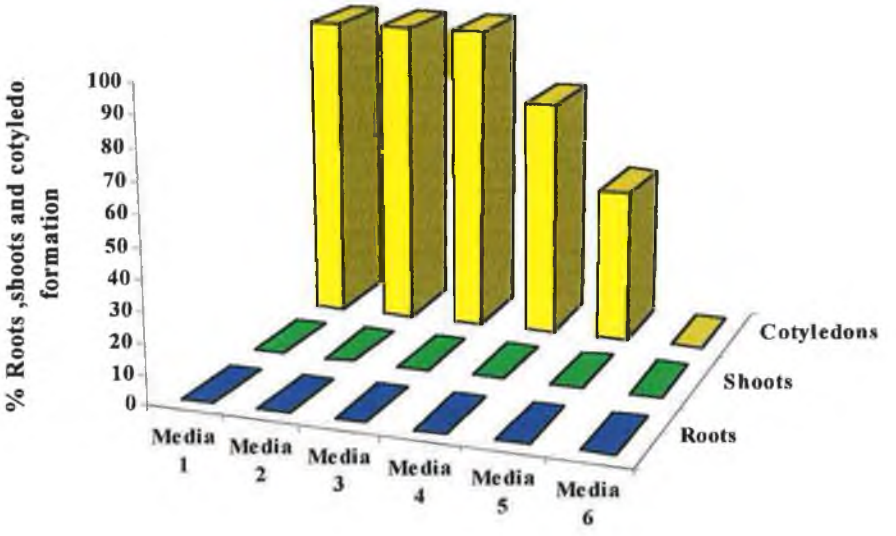
Figure 5.22 shows % root, shoot and cotyledon formation on Oak embryogenic callus following a 7 week incubation period on all six P24 maturation media. Graph A shows the results of plates which were incubated in the light and Graph B shows plates which were cultured in the dark. Results indicated that culture in the light gave significantly better regeneration results than culture in the dark. Culturing in the dark did

not yield any root or shoot growth whereas exposure to the light yielded root proliferation in media 1 and 2 though at a relatively low percentage and shoot establishment in media 1 and 5. Both root and shoot establishment was achieved in medium1.

Figure 5.23 shows regeneration following transfer to P24 medium 1 in the light. Results were extremely favourable exhibiting almost 50% root formation and 30% shoot formation (Plate 5.4). Cotyledon formation was also recorded at a high percentage of 70% (Plate 5.5). These results are further discussed in the discussion section on page 221 and conclusions drawn on page 228.



A



B

Figure 5.22
Maturation of Oak embryogenic callus on P24 maturation medium. Oak embryogenic callus was cultured on P24 medium plus 0.9 μM BAP for 5-7 weeks in the light. Cultures were then transferred to P24 maturation media 1-6 and cultured for a further 7 weeks in the light (A) or in the dark (B). Percentages were calculated from 5 plates for each media type with each plate containing 5 pieces of oak callus.

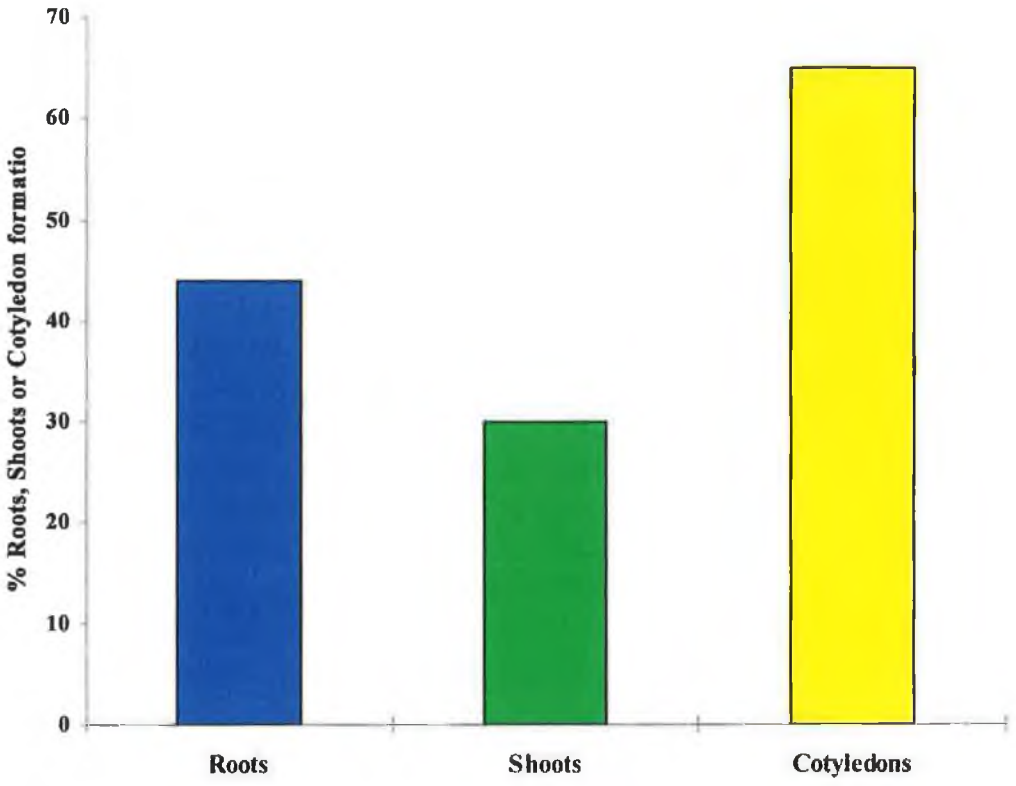


Figure 5.23

Maturation of Oak embryogenic callus. Graph shows percentage root, shoot and cotyledon formation following transfer of all cultures from P24 maturation media 1-6 after a 7 week culture period to P24 maturation medium type 1. All cultures were grown in the light for a further 5-7 weeks. Percentages were calculated from a total of 60 plates each containing 5 oak callus pieces.

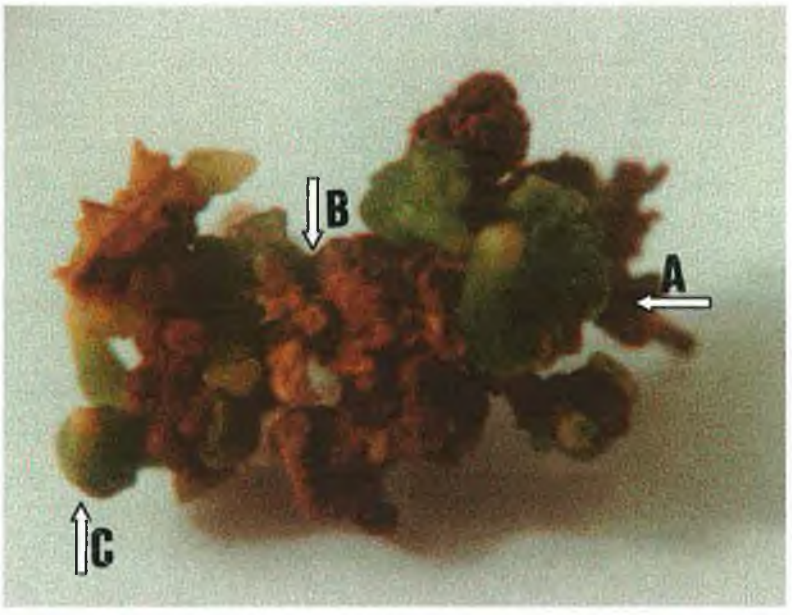


Plate 5.4

Oak embryogenic callus following maturation on P24 medium plus 0.9 μM BAP for 5 weeks in the light with transfer to P24 maturation medium 1 in the dark for a further 7 weeks and final transfer to fresh P24 maturation medium 1 in the light for a further 7 weeks. The plate indicates the presence of cotyledon formation and greening (A). Cell necrosis (B) and callus greening (C) were also exhibited. Bar = 5mm.

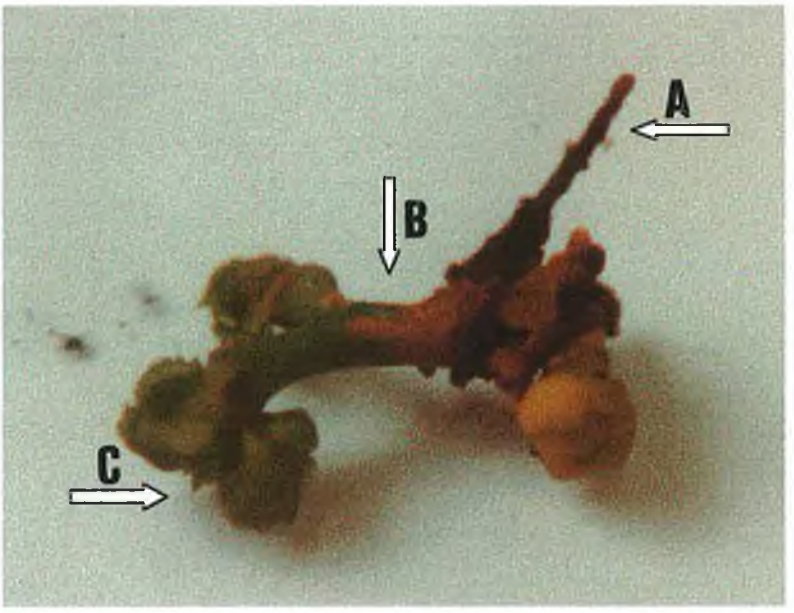


Plate 5.5

Oak callus following maturation on P24 medium plus 0.9 μM BAP for 5 weeks in the light with transfer to P24 maturation medium 1 in the dark for a further 7 weeks and a final transfer to fresh media type 1 and culture in the light for 7 weeks. The plate indicates root (A) and shoot (B,C) development. Bar = 5mm.

5.2.7 Maturation of Sitka Spruce (*Picea sitchensis* (Bong.) Carr.) Embryonal Suspensor Masses

Maturation protocols for Sitka Spruce embryonal suspensor masses have previously been published (Krogstrup, 1988). However growth of Sitka Spruce embryonal suspensor masses can vary in different environments and therefore it was important confirm that regeneration could be achieved from our ESMs. Successful regeneration experiments would mean that we could then regenerate plantlets from our transformed ESMs.

Following 3–4 subcultures on somatic embryo initiation and maintenance medium ESMs (Plate 5.6) were transferred to Sitka Spruce somatic embryogenesis, embryo development medium. Embryonal suspensor masses were cultured on this medium for 6–8 weeks in the dark. Developing somatic embryos were seen on the ESMs after 6–8 weeks and were heart shaped and yellow in appearance. Each developing embryo was transferred with a sterile watchmakers forceps to plates containing Sitka Spruce embryogenesis, embryo germination medium. Each plate was cultured in the dark for 5–7d and then taken into the light for the next 5–7 weeks. The individual plantlets were separated out as they developed and placed on fresh germination medium every 6–8 weeks.

Figure 5.24 shows percentage root and shoot production on germinating Sitka Spruce embryos. Results showed that shoot formation (90%) was almost twice that of root formation (50%). However the number of germinating embryos present with both roots and shoots (50%) (Plate 5.7) was sufficiently satisfactory.

Transformation of Sitka Spruce ESMs involved the addition of cefotaxime into the medium in order to inhibit continued growth of *Agrobacterium* following a co-cultivation period. The effect of cefotaxime on the growth of ESMs has already been investigated and shown to have no affect at levels as high as 1000 $mgdm^{-3}$. However we also needed to assess the affects of high levels of cefotaxime on the regeneration of ESMs. The next experiment investigates these affects.

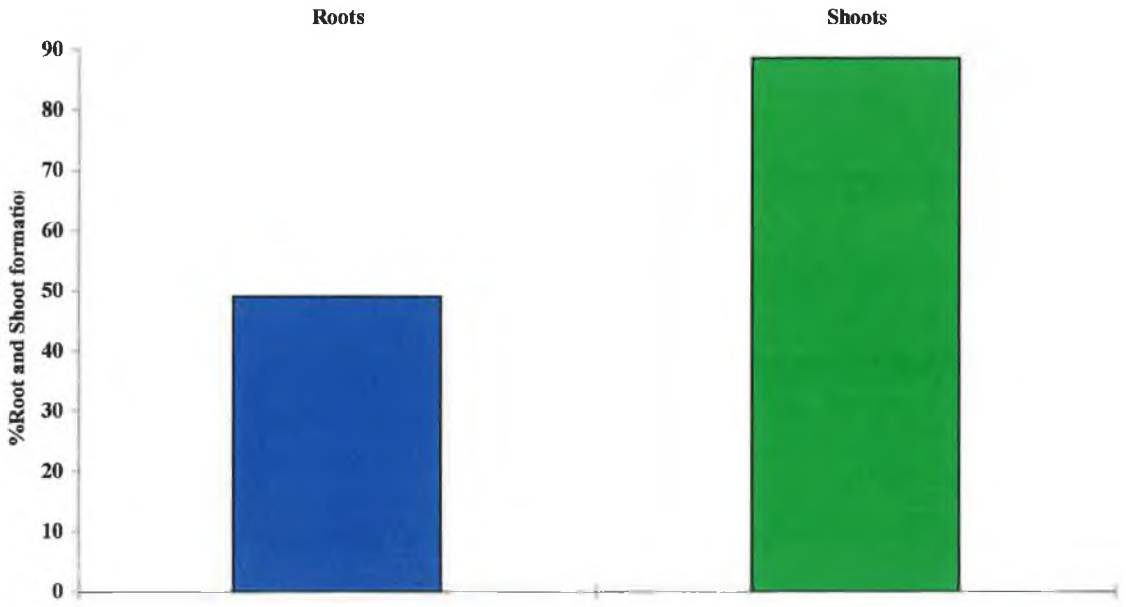


Figure 5.24

Maturation of Sitka Spruce germinating embryos. Figure shows percentage root and shoot formation on sitka spruce germinating embryos. Embryos were germinated on sitka spruce embryogenesis, embryo germination medium for 15 weeks in the light. Percentages were calculated from 60 plates each containing between 5–10 germinating embryos.

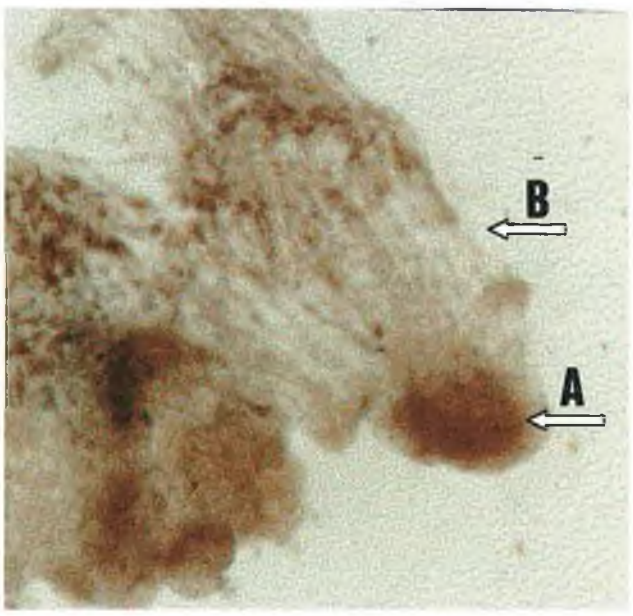


Plate 5.6

Sitka Spruce (*Picea sitchensis* (Bong) Carr.) ESMs showing stage 1 somatic embryos in sitka spruce embryogenesis, embryo development medium. The presence of an embryogenic head (A) and elongated suspensor cells (B) are evident from the plate.

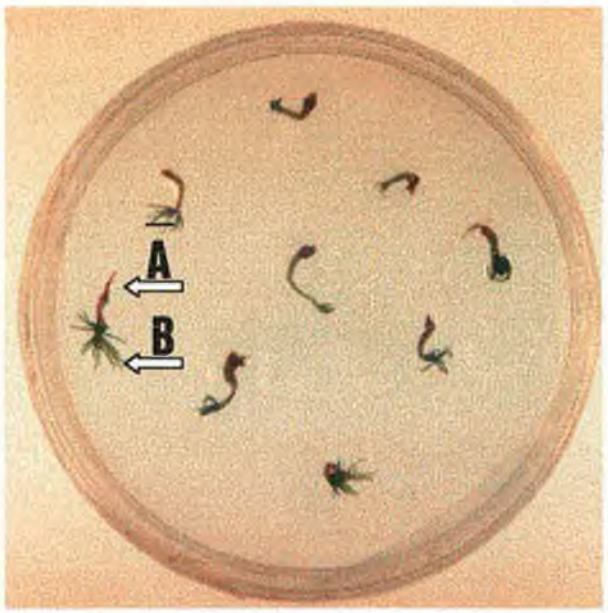


Plate 5.7

Germination of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses on embryo germination medium. Plate shows the root (A) and shoot (B) proliferation. 9 cm Petri dishes.

5.2.8 Regeneration of Sitka Spruce (*Picea sitchensis* (Bong.) Carr.) Embryonal Suspensor Masses in Varying Concentrations of Cefotaxime Antibiotic

Sitka Spruce embryonal suspensor masses were regenerated in the presence of varying concentrations of cefotaxime antibiotic. Sarma *et al.*, 1995 reported a significant reduction in the number of maturing somatic embryos of Sitka Spruce in the presence of cefotaxime antibiotic.

Sitka Spruce embryogenesis, embryo development medium and embryo germination medium were prepared as normal. Cefotaxime at concentrations varying from 0 $mgdm^{-3}$–1000 $mgdm^{-3}$ were added to the autoclaved medium. Embryonal suspensor masses were regenerated in the same manner as above.

Figure 5.25 shows the number of developing embryos present on embryo development medium. Results showed a reduction in the number of developing embryos in cefotaxime containing medium compared to numbers present in the absence of cefotaxime. However, the number of developing embryos on cefotaxime concentrations up to 700 $mgdm^{-3}$ are relatively high at about 1/5 of that produced at 0 $mgdm^{-3}$ cefotaxime. The number of developing embryos produced on medium containing cefotaxime concentrations above 700 $mgdm^{-3}$ were however significantly lower than at 700 $mgdm^{-3}$.

Results showed that below 700 $mgdm^{-3}$ cefotaxime (Fig. 5.26) there was no effect on shoot formation. Shoot formation at 800-1000 $mgdm^{-3}$ was reduced but not significantly. The growth of roots was recorded as 50% for 0 $mgdm^{-3}$ cefotaxime, which was similar to results recorded in the previous experiment. However, root formation for

100 $mgdm^{-3}$ and 300 $mgdm^{-3}$ was recorded in less than 10% of developing embryos. This level of root formation was however increased for all other cefotaxime concentrations ranging from 20%-60%.

Results on the affect of cefotaxime showed that it decreases the numbers of developing embryos produced. Further germination of these embryos was successful and all embryos developed into plantlets complete with roots and shoots. but does not affect the subsequent development of roots and shoots. Therefore, it can be concluded that the use of cefotaxime for the elimination of *Agrobacterium* following co-cultivation was advisable as its effects on growth and regeneration of Sitka Spruce ESMs was negligible.

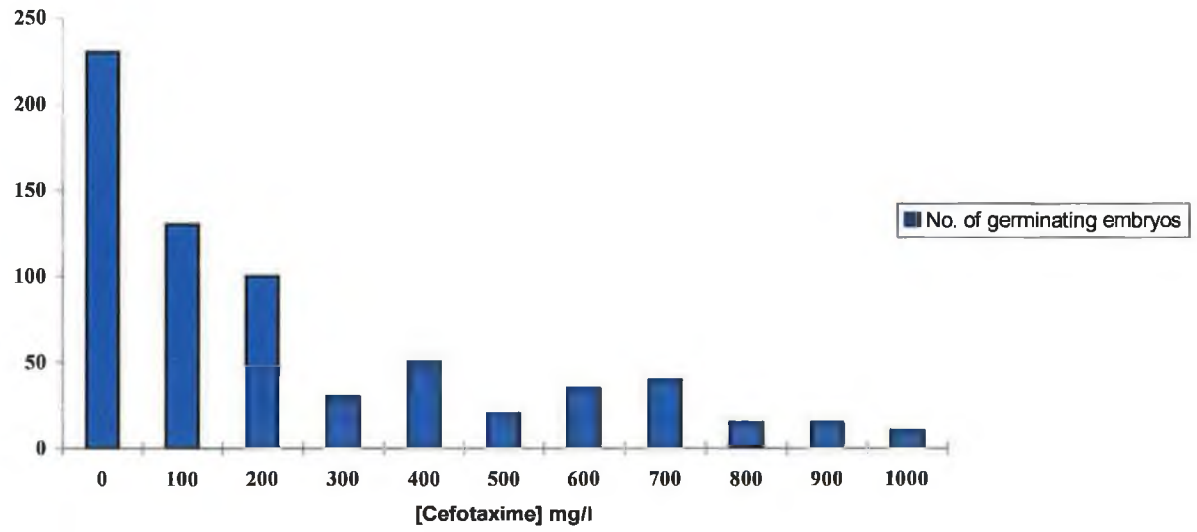


Figure 5.25

Maturation of Sitka Spruce embryonal suspensor masses on Sitka Spruce embryo development medium. Figure shows number of developing embryo formation on Sitka Spruce embryo development medium plus varying concentrations of cefotaxime antibiotic (0 $mgdm^{-3}$–1000 $mgdm^{-3}$). Embryo suspensor masses were cultured for 7 weeks in the dark. Developing embryos were excised aseptically from 11 embryo suspensor masses per cefotaxime concentration and counted before culturing on embryo germination medium.

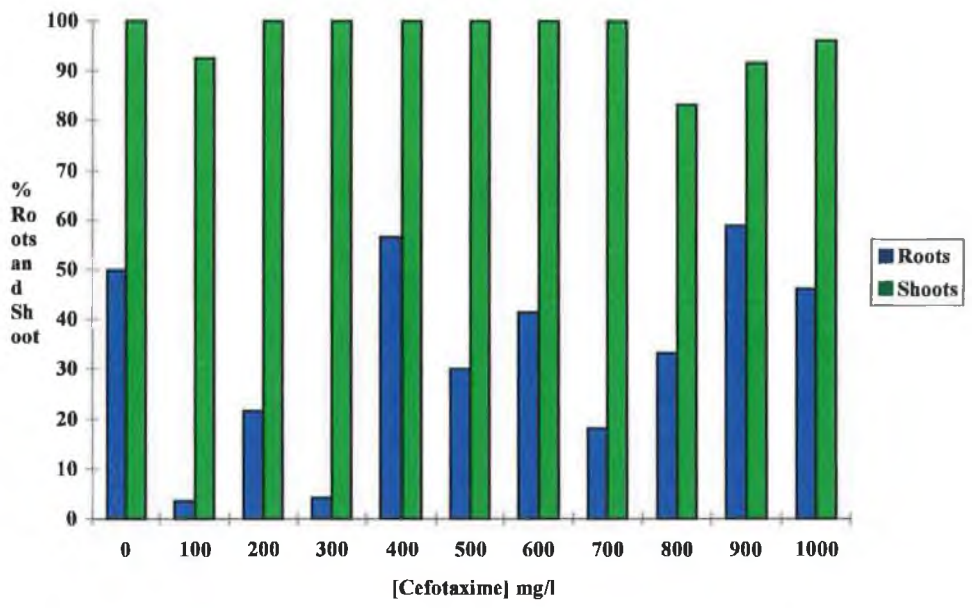


Figure 5.26

Maturation of Sitka Spruce developing embryos on Sitka Spruce embryo medium. Figure shows percentage root and shoot formation from developing embryos on embryo developing medium plus varying concentrations of cefotaxime antibiotic. Developing embryos were cultured on this medium for 15 weeks in the light. Percentages were calculated from between 18–26 developing embryos per cefotaxime concentration.

5.3 Discussion

A serious problem of woody plant regeneration via somatic embryogenesis is either the absence or low frequency of somatic embryo conversion into plantlets. Germination of somatic embryos is more often stimulated by alteration of physical conditions and medium changes. The addition of exogenous hormones has proved to be most successful.

The development of somatic embryos is often blocked after the formation of cotyledons (Chalupa, 1995). Therefore for the development of somatic embryos into plants various treatments were tested. The alteration of physical conditions appeared to induce embryo germination. It is well established that desiccation plays a role in switching zygotic embryos from embryogenic to germinative stage (Kermode *et al.*, 1977). Oak embryogenic cultures were desiccated with increasing concentrations of sorbitol (0%-12%) on ½ MS and WPM medium. Results showed 0% shoot formation and a low frequency root formation (15%) on transfer of cultures to WPM and ½ MS in the light. Chalupa, 1995 reported stimulation of *Q. petraea* embryo conversion by desiccation and exposure to cold followed by transfer to WPM containing low concentrations of BAP (0.44 μM). However, high osmoticum and chilling treatment had no effect on germination frequency *of Q. acutissima* embryos (Kim *et al.*, 1994). Ostrolucka *et al.*, 1996 reported *Q. robur* embryo germination on medium containing 0.1 $mgdm^{-3}$ BAP after previous culture on WPM hormone-free medium supplemented with 6 % sorbitol. Osmotic stress can also be induced by increasing the agar concentration. Experiments on P24 maturation media with increased agar concentrations showed increased embryo germination. The frequency of root (15%-40%) and shoot (15%–100%) proliferation was greatly improved with the use of agar as an osmoticum and previous culture on P24 medium plus 0.9 μM BAP or hormone

free P24 and culture in the light. Eva Wlihelm (per comm) achieved an 80% increase in the maturation of *Q. robur* somatic embryos by a partial desiccation treatment with 0.8–1 % agar during light culture conditions. The maturation phase was an important step for the fate and survival of somatic embryos.

The addition of exogenous medium components also proved beneficial in the germination of embryo cultures. Abscisic acid has been used in a number of maturation protocols (Kim *et al.*, 1994; Cuenca *et al.*, 1999). Addition of ABA concentrations ranging from 0–100 μM in the presence of sorbitol desiccation had little effect on root and shoot proliferation of Oak embryo cultures. Anthocyanin production was recorded at a higher percentage in the presence of ABA in the culture medium. Shoot formation was absent and root formation was recorded in the presence of ABA however at a higher frequency on cultures grown on medium containing 0 μM ABA. Kim *et al.*, 1994 reported root and shoot formation on medium supplemented with 0.1 $mgdm^{-3}$ ABA on MS medium. Embryos matured on medium supplemented with 8% sucrose and 2.7 $mgdm^{-3}$ ABA showed root development and root and shoot development at frequencies up to 43% and 36% respectively (Cuenca *et al.*, 1999). Germination of *Q. robur* embryos occurred on media with ABA and GA_3 (0.2 $mgdm^{-3}$) after previous culture on WPM hormone free medium supplemented with 6% sorbitol (Ostrolucka *et al.*, 1996).

Maturation of Sitka Spruce embryonal suspensor masses is greatly affected by the presence of exogenous ABA. Sitka Spruce embryo development medium was supplemented with ABA (50 $mgdm^{-3}$) and yielded up to 230 yellow globular embryos. Krogstrup, 1988 observed that the use of ABA in maturation medium resulted in synchronised maturation of Sitka Spruce somatic embryos. The best maturation in terms of morphological characters he reported was obtained on medium with ABA as the sole

growth regulator. Boulay *et al.*, 1988 reported that in ESMs of *Picea abies* ABA inhibited the cleavage process in ESMs and stimulated development of proembryos. On medium without ABA fewer proembryos developed into mature embryos. Addition of ABA to coniferous tissue has also been investigated by Gupta and Durzan, 1987a who used 0.5 μM ABA to promote embryo maturation from protoplasts of Loblolly pine and by Durzan and Gupta, 1987 who reported the promotion of embryo maturation by 0.5 μM ABA in embryogenc suspensions cultures of Douglas Fir. Von Arnold and Hakmann, 1988 have showed that maturation of Norway spruce embryos occurred after treatment with 7.6 μM ABA.

The addition of exogenous cytokinins to the medium in particular benzylaminopurine proved necessary in the germination of Oak embryo cultures and in particular for shoot proliferation. Our experiments utilised BAP alone or in combination with exogenous auxins (2,4-D, GA_3, IBA). In all experiments induced embryoids were always transferred to mediums with a low BAP concentration (0.2 $mgdm^{-3}$–0.6 $mgdm^{-3}$) or initially cultured on medium with low BAP (0.9 μM) concentrations. Results showed that culture of Oak embryogenic callus on WPM containing 0.5, 1 and 2 $mgdm^{-3}$ BAP and subsequent transfer to WPM plus 0.2–0.6 $mgdm^{-3}$ BAP in the light produced high numbers of secondary cotyledons (60%-100%) and callus greening (60%-100%). There was however an absence of both root and shoot proliferation. Results on P24 medium showed that in the majority of cases culture on medium plus 0.9 μM BAP and transfer to P24 maturation media 1–6 yielded much higher root and shoot proliferation in contrast to cultures transferred from hormone-free P24 medium. Chalupa, 1987a, 1990a, reported that germination of *Q. robur* somatic embryos and formation of plantlets occurred rarely on medium containing high concentrations of cytokinins. However, transfer of Oak

embryogenic tissue with induced embryoids to WPM containing a reduced (0.2–0.6 $mgdm^{-3}$) cytokinin concentration, culturing them in the light led to callus greening and further development of embryoids. However, he found that the frequency of conversion to plantlets was low and the majority of cultures produced somatic embryos which did not germinate. The germination of *Q. acutissima* somatic embryos into plantlets was reported to be stimulated on WPM containing a low concentration of BAP (Sasaki *et al.*, 1988: Shoyama *et al.*, 1992). *Q. petraea* embryogenic cultures were germinated on MS containing 2.5 μM BAP (Jorgenson, 1988). Chalupa, 1988 suggested that shoot proliferation of *Q. robur* cultures was greatly dependent on the type and concentration of cytokinin. The number of shoots recorded on medium with low concentrations of cytokinin was significantly higher than on media lacking cytokinin. He reported that low BAP concentrations ranging from 0.1–0.2 $mgdm^{-3}$ promoted shoot proliferation. Increasing BAP concentration to 0.4–0.6 $mgdm^{-3}$ produced the largest number of shoots. Increasing the concentration of BAP to 2 $mgdm^{-3}$ produced short shoots with a large callus forming at the basal part of the shoot. Vieitez *et al.*, 1985 showed that inclusion of BAP in medium significantly effected growth and proliferation of Oak shoots.

The introduction of auxins into maturation mediums in combination with a source of cytokinin has proved favourable in the regeneration of somatic embryos (Kim *et al.*, 1997; Chalupa, 1990; Ostrolucka *et al.*, 1996). Exogenous auxins used most frequently are IBA, GA_3 and 2,4-D. Results presented show the effects of all three auxins in concentrations ranging from 0.1 $mgdm^{-3}$–1 $mgdm^{-3}$ in combination with BAP for regeneration of Oak embryogenic callus. For all combinations both root and shoot proliferation failed to occur. Inclusion of IBA into WPM resulted in both callus greening and cotyledon formation similar to levels observed in its absence from the medium. Regeneration on ½ MS medium was less successful with a reduction in cotyledon

formation to 0% for some combinations and an absence of callus greening on transfer of all cultures from ½ MS medium plus BAP (0.5 $mgdm^{-3}$, 1 $mgdm^{-3}$, 2 $mgdm^{-3}$) plus 1 $mgdm^{-3}$ IBA with the exception of transfer from 2 $mgdm^{-3}$ BAP plus 1 $mgdm^{-3}$ IBA to WPM plus 0.6 $mgdm^{-3}$ BAP where it was recorded at 100%. Chalupa, 1990 reported that development of embryogenic cultures of *Q. robur* was best initiated on MS medium and WPM containing BAP (1 $mgdm^{-3}$) plus IBA (0.1 $mgdm^{-3}$,1 $mgdm^{-3}$).

Chalupa also reported that 2,4-D appeared to be ineffective for stimulation of somatic embryogenesis in *Q. robur* cultures and that substitution of IBA for 2,4-D led to the stimulation of callus formation. Results presented indicate that cultures transferred from ½ MS medium with BAP (0.5 $mgdm^{-3}$,1 $mgdm^{-3}$, 2 $mgdm^{-3}$) plus 2,4-D (0.5 $mgdm^{-3}$, 1 $mgdm^{-3}$) to WPM plus BAP (0.2 $mgdm^{-3}$–0.6 $mgdm^{-3}$) resulted in complete inhibition of callus greening and a slight reduction in cotyledon formation. Gingus *et al.*, 1989 reported the production of high embryoid numbers from explants of *Quercus* cultured on modified MS medium supplemented with 1 $mgdm^{-3}$ 2,4-D and 1 $mgdm^{-3}$ BAP. The use of 2,4-D has proven to be non-beneficial in the regeneration of other woody species. Bonneau *et al.*, 1994 reported the presence of somatic embryos in the European spindle tree (*Euonymus europaeus* L.) in the presence of IAA and NAA in the induction medium but never with IBA or 2,4-D.

The substitution of IBA and 2,4-D with GA_3 proved to be of little significance in our results. GA_3 (0.5 $mgdm^{-3}$, 1 $mgdm^{-3}$) in combination with BAP (0.5 $mgdm^{-3}$, 1 $mgdm^{-3}$, 2 $mgdm^{-3}$) on ½ MS medium and subsequent transfer to WPM plus BAP (0.5 $mgdm^{-3}$, 1 $mgdm^{-3}$, 2 $mgdm^{-3}$) again showed a reduction in cotyledon formation and the inhibition of callus greening. Chalupa, 1990 reported that embryogenic tissue of *Q. robur* produced on MS medium and WPM containing BAP or BAP plus GA_3 could easily be maintained and subcultured and that within 8-10 weeks globular structures developed in 60-70% of

cultures. Addition of 0.2 $mgdm^{-3}$ BAP or 0.1 $mgdm^{-3}$ BAP plus 0.1 $mgdm^{-3}$ GA_3 to WPM results in effective epicotyl formation in *Q. acutissima* cultures (Kim *et al.*, 1997). The addition of GA_3 to growth medium has been reported to enhance the embryogenic potential of a number of plants such as fennel (Hunaut *et al.*, 1995) and *Camellia japonica* hypocotyls (Vieitez and Bariela, 1990). In some systems GA_3 treatment has been reported to enhance the germination of somatic embryos into plantlets (Culafic *et al.*, 1987; Chang and Hsing, 1980). Both 2,4-D and BAP have been reported to enhance individual somatic embryo formation in Sitka Spruce (Krogstrup, 1988).

Thidiazuron has been reported as being among the most active cytokinin like substances in woody plant tissue culture. Low concentrations (< 1 μM) can induce greater axillary proliferation than many other cytokinins. It has however been suggested that TDZ is more active in stimulating adventitious shoot formation than somatic embryogenesis (Heutteman *et al.*, 1993). Our results with TDZ were in total contrast to previous reports. Results showed no root or shoot proliferation and complete absence of embryoid or cotyledon formation. Navarrette *et al.*, 1989 observed that 3 or 10 μM TDZ stimulated axillary shoot proliferation in white ash. Chalupa, 1988 showed that TDZ effected elongation and morphology of Oak shoots very significantly. The presence of TDZ he reported at very low concentrations (0.001 $mgdm^{-3}$–0.004 $mgdm^{-3}$) promoted shoot formation.

The presence of activated charcoal appeared to increase Oak embryo regeneration on P24 maturation medium 1. Results showed a substantial increase in the production of roots and shoots together and a high percentage of cotyledon formation. The inclusion of 2% AC on ½ MS medium and WPM seemed ineffective in regeneration of Oak embryogenic cultures with results yielding 0% root and shoot proliferation. Jorgenson,

1988 reported that the embryogenic potential of *Q. petraea* was greater if activated charcoal was added to the MS medium plus 2.5 μM BAP otherwise embryos turned to calli. The presence of AC in Sitka Spruce embryo development medium increases the production of germinating embryos.

The growth of cultures in the presence or absence of light in the culture room had a large effect on the regeneration of Oak embryogenic cultures. Transfer of cultures from the light to the dark on ½ MS medium or WPM for the final stages of germination resulted in all cases in the absence of callus greening and a reduction in cotyledon formation. On P24 medium transfer of cultures from the light to the dark resulted in most cases in a reduction of root and shoot formation. Wilhelm (per comm) suggested that for *Q. robur* light conditions favoured development of somatic embryos in general more than cultivation without light. Survival rate (%) Oak somatic embryos after cultivation in the dark was reported to be at least 10% below Oak somatic embryos cultured in the light after desiccation treatment.

We showed that cefotaxime antibiotic had no effect on the growth of Sitka Spruce ESMs in liquid culture up to 1000 $mgdm^{-3}$ in chapter 3. Sarma *et al.*, 1995 reported a 20% reduction in stage 2 somatic embryo production in Sitka Spruce in the presence of cefotaxime with a subsequent reduction of 80%(stage 3 embryos) and 60% (stage 4). Results however showed that high (800–1000 $mgdm^{-3}$) concentrations of cefotaxime reduced the number of germinating embryos produced on Sitka Spruce embryo development medium by about 10% compared to 0 $mgdm^{-3}$ cefotaxime. However it did not effect the subsequent root and shoot proliferation on these embryos on embryo germination medium. Drake *et al.*, 1997 also reported that cefotaxime had no effect on tissue growth of Sitka Spruce. However their results reported that cefotaxime inhibited somatic embryogenesis.

5.4 Conclusion

To conclude Oak regeneration experiments performed on full or half strength Murashige and Skoog medium yielded poor results for root and shoot proliferation in the presence of all external growth hormones. Regeneration on WPM medium proved slightly better but did not achieve the production of whole plantlets. Results on P24 maturation media produced results of a much higher standard and in particular results yielded on P24 maturation medium 1 were particularly promising with the growth of both roots and shoots on the same germinating embryos. It was evident that culture in the light was more beneficial for both greening and cotyledon formation. The use of 0.9 μM BAP in P24 maturation medium proved to be advantageous for callus rooting and shooting. However, external hormones IBA, 2,4-D, GA_3 and TDZ had a negligible effect on regeneration. Regeneration of Sitka Spruce embryonal suspensor masses was more successful with the production of whole plantlets complete with roots and well established shoots.

The next chapter examines the production of single cells from both Oak and Sitka Spruce embryonal cultures

Optimized Protocol for the Maturation and Regeneration of Oak Embryogenic Callus Cultures

Preparation of 1 dm^3 P24 medium (0.9 µM B.A.P.)

↓ 20cm^3 dispensed into sterile 9cm Petri dishes

5 pieces of oak callus cultures plated onto each

↓ growth room, light, 5-7wks

Preparation of 500 cm^3 of P24 maturation media 1+6

↓ 20 cm^3 dispensed into steril 9 cm Petri dishes

From previous 30 plates, transfer 15 plates of callus to each P24 maturation media 1+6, ½ of each media type are cultured in light and ½ in the dark

↓ 7 wks in growth room

Prepare 1 dm^3 P24 maturation medium type 1

↓ 20 cm^3 is dispensed into sterile 9 cm Petri dishes

All cultures transferred to P24 maturation medium type 1

↓ light, growth room

Cultures were subcultured every 5 wks onto fresh P24 maturation medium and observed the development of roots and shoots.

Chapter 6

Production, Regeneration and *Agrobacterium*-mediated Transformation of Oak (*Quercus robur* L.) and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Single Cell cultures.

6.1 Introduction

With the development of plant biotechnology protoplast culture has become widely applied to cell biology and molecular biology because of its importance in gene transformation, somatic hybridisation and variation of somatic clones. Although progress has already been made in many crop plants the biotechnology of forest trees is behind in many respects because of its long regeneration time (Qiao *et al.*, 1998).

Regeneration of shoots fromprotoplasts is a useful tool for the genetic manipulation and improvement of plants. There are only a few forest tree species in which plant regeneration from protoplasts have been successful *Pinus glauca* (Attree *et al.*, 1987), *Pseudosuga meniesii*, *Pinus taeda* (Gupta and Durzan, 1987; Gupta *et al.*, 1988).

Direct gene transfer using protoplasts has been successful to obtain stable transgenic plants (Koop *et al.*, 1996; Dhir *et al.*, 1992; Lazzeri *et al.*, 1991; Shimamoto *et al.*, 1989). In the majority of studies, researchers used embryogenic cell suspension cultures as a source of material. Therefore the use of embryogenic cultures as a source of protoplasts offers a unique opportunity to regenerate plants.

The following chapter examines the potential of single cells of Oak (*Quercus robur* L.) and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) from embryogenic suspension cultures. This chapter investigates the growth of embryogenic cultures and single cell cultures in 24 well multi-well plates and the capacity to regenerate microcalli from cultures of single cells. Finally, we looked at the possibility of *Agrobacterium*-mediated transformation of single cell cultures of Oak (*Quercus robur* L.).

6.2 Results

The production of single cells from embryogenic suspension cultures of both Oak and Sitka Spruce was investigated. The ability to produce single cells and monitor their growth in multi-well plates could be seen as another method of assessing embryogenesis in both tree species. Production of single cell cultures would lead to a second source of material for transformation experiments.

The following chapter assesses ways of producing single cells from embryogenic cultures.

6.2.1 Manufacture of a Plate Reader for Continuous Monitoring of Cell Growth

In order to quantitatively measure growth of isolated single cells in culture, it was necessary to use a device that could measure growth in small volumes (1-2 cm^3) with a high accuracy and precision. This was achieved using a 24 well multi-well plate reader described in section 2.3.2. A photograph of the reader is shown in Plate 6.1.

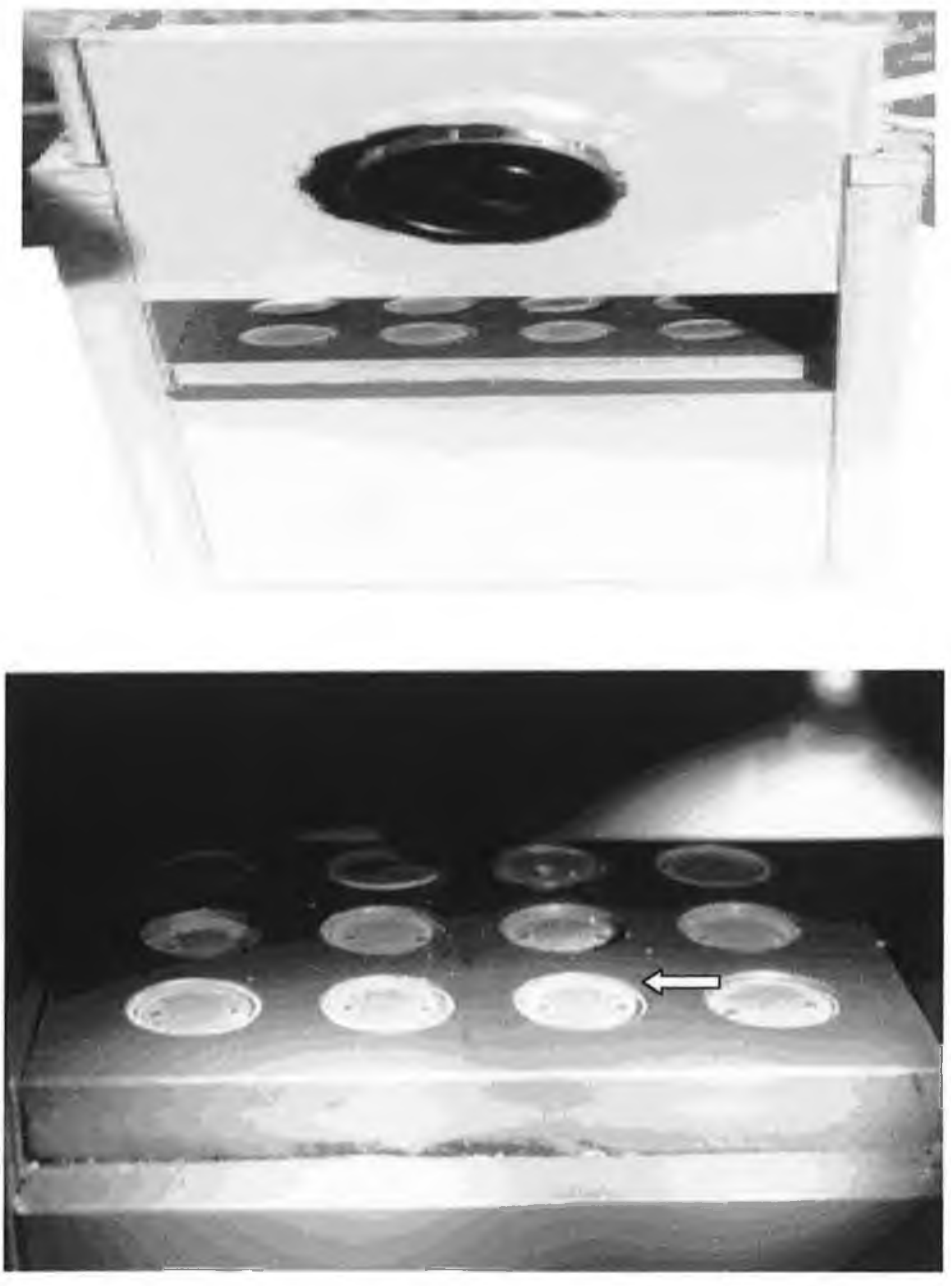


Plate 6.1

External (top) and internal (bottom) view of 24 well plate reader used to continuously monitor cell growth in the plant culture room. The arrow shows the position of the LDR over which each well of the plate sits.

6.2.2 Monitoring of Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Embryonal Suspensor Masses in the 24 well Multi-Well Plate Reader

Growth of Sitka Spruce embryonal suspensor masses in 24 well multi-well plates needed to be assessed, firstly to see if the plate reader was working and secondly to assess its suitability for monitoring the growth of embryonal suspensor masses.

From a freshly subcultured flask of Sitka Spruce embryogenic suspension cells, 1 cm^3 aliquots were taken and placed in the individual wells of a sterile 24 well multi-well plate. The 24 well plate was sealed with a single layer of parafilm being careful not to splash the cells onto the lid of the plate. Splashing would have led to inconsistencies in the readings. The plate was then inserted into the plate reader which was located on top of a gyratory shaker set at 100 r.p.m. in the plant culture room. The plate reader was set to continuously read the OD ($1A_{565nm}$ = $25gdm^{-3}$ fr. wt)of the plant cells and readings were saved periodically onto a floppy disc to avoid loss of results. A number of wells were selected (Fig. 6.1) and the mean of their readings taken for certain time intervals. The graph shows that there was an increase in cell growth for all wells from 0h-250h. Plates were removed from the plate reader and checked for bacterial contamination on a twice daily basis to ensure that increases in readings were not due bacterial comtamination. However, it was observed that beyond this period the plate reader was not suitable for reading these cells as the well volume restricted continued growth. It was observed that the cells became highly aggregated.

Results here show that the plate reader was working effectively and could be used for monitoring cell growth. It appears that it would be more suitable for monitoring single cell growth due to the limited capacity of the wells in the multi well plate. Cultures grow equally well in light and dark so light does not appear to be an important for growth of

embryo cultures. Green light is at a minimum for phytochrome. The next section investigates the production of both Oak and Sitka Spruce single cells from embryogenic cultures.

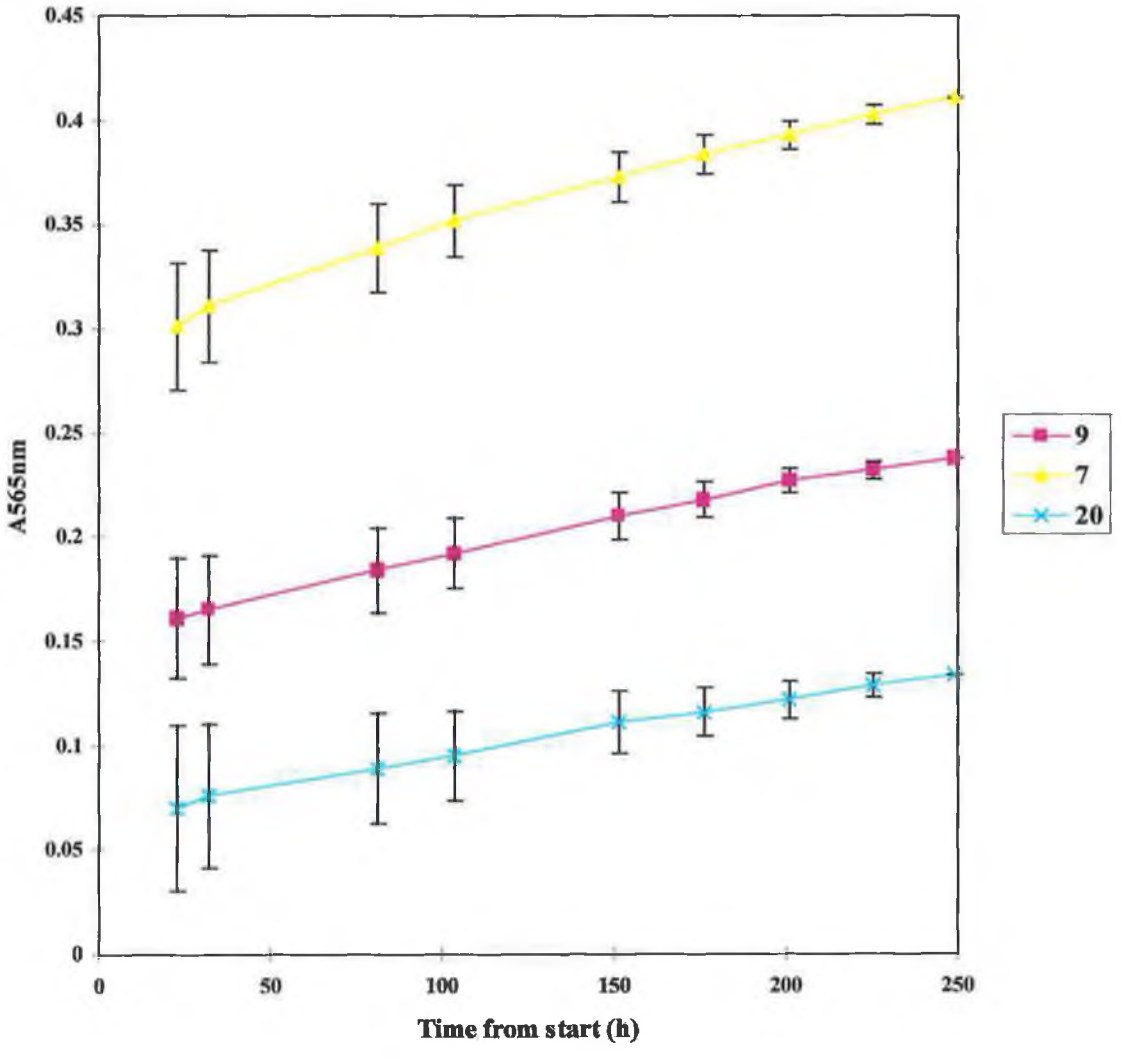


Figure 6.1

Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) embryonal suspensor masses in a 24 well-multi well plate. The mean growth of cells (OD) in wells 7, 9 and 20 was calculated and graphed. The experiment was carried out in duplicate and standard errors of the mean are included.

6.2.3 Pectinase Digestion of Oak (*Quercus robur* L.) and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Embryogenic Suspension Cultures

In order to produce single cells the first factor investigated was the use of pectinase to release embryogenc heads from the suspensor cells.

Aliquots of 5 cm^3 of both MS medium and Sitka Spruce embryogenesis initiation media were aseptically transferred to sterile 20 cm^3 Universal bottles. Pectinase concentrations of 0 gdm^{-3}, 0.01 gdm^{-3}, 0.03 gdm^{-3}, 0.05 gdm^{-3}, 0.07 gdm^{-3} and 0.1 gdm^{-3} were added to the media. The mixtures were then filter sterilized. The contents of 7d old Oak and Sitka Spruce suspension cultures were sieved through 500 μm mesh. The cell retentate was weighed in a sterile petri dish and to each Universal bottle 0.5 g fr. wt. of cells were added. The cells were vortexed at full speed for 40s and placed on a shaker at 100 r.p.m in the plant culture room for 9h. Samples were then removed and 200 μl aliquots were observed under 10 X magnification and images recorded using the OPTIMAS 6 image analysis programme.

The progression in cell digestion can be seen for Oak in Plate 6.2. At 0 gdm^{-3} pectinase cells were highly aggregated embryogenic clusters however, at increased enzyme concentrations it could be clearly seen that individual cells and multi cell clusters started to dissociate themselves from the main cell clumps. At concentrations above 0.03 gdm^{-3} pectinase, virtually all of the cell clumps were digested and only single cells remained in culture after 9h.

Pectinase digestion of Sitka Spruce embryonal suspensor masses can be seen in Plate 6.3. Again as with Oak there was an obvious progression from 0 gdm^{-3}–0.1 gdm^{-3} pectinase. Pectinase concentrations of 0.01 gdm^{-3} and 0.03 gdm^{-3} appeared to simply

separate the stage 1 embryos from each other but left the basic structure of embryogenic head with elongated suspensor cells attached intact. Increasing the concentration to 0.05 gdm^{-3} resulted in the detachment of the majority of the suspensor cells from the embryogenic head. To dissociate single cells from the embryogenic head the pectinase concentration needed to be increased to 0.07 gdm^{-3}–0.1 gdm^{-3}. However, although single cells were now evident they tended to be mixed with cell fragments and suspensor cells. In order to free cultures of cell debris a method of fractionation needed to be investigated. The following experiment investigates this notion of cell fractionation.

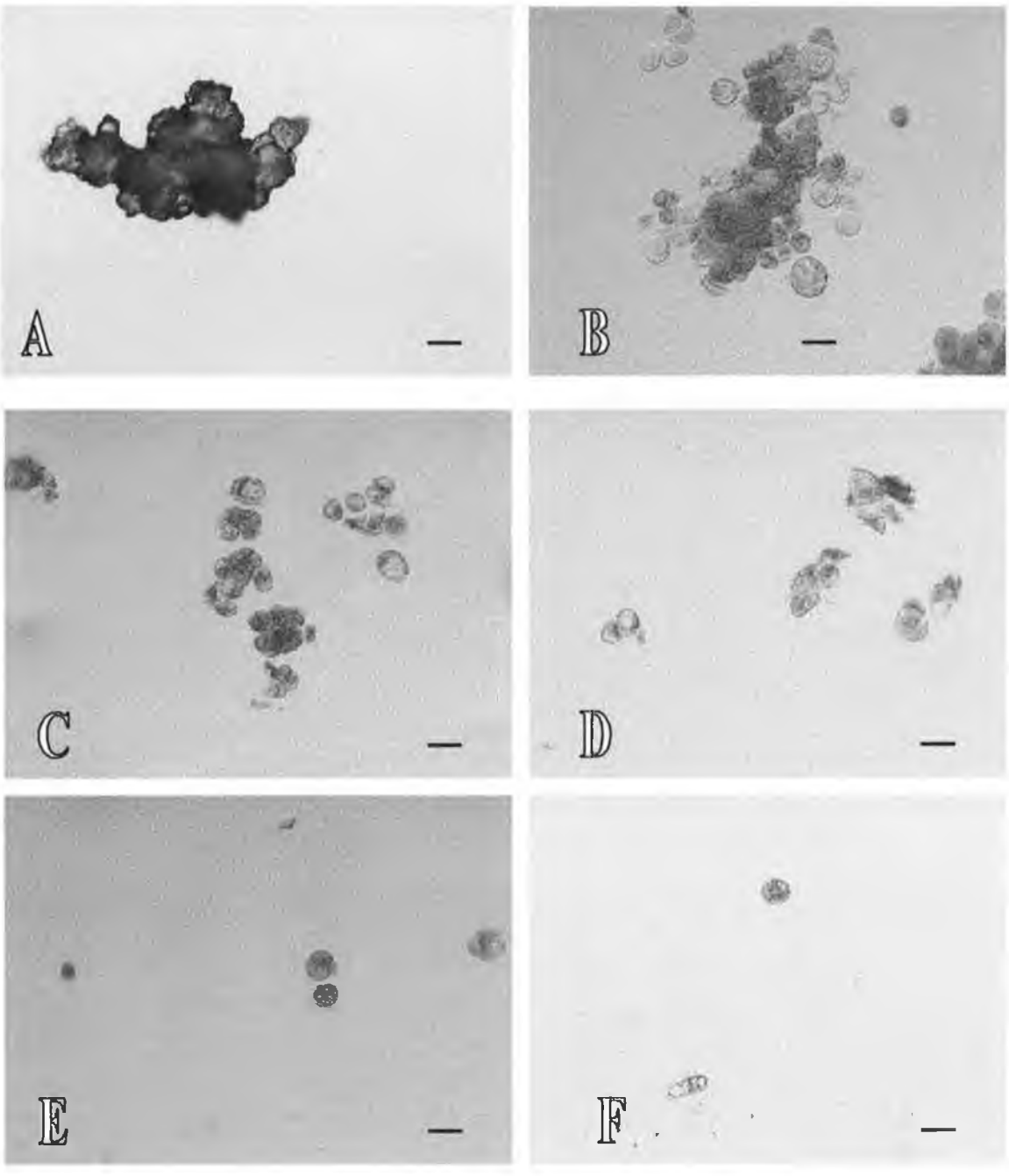


Plate 6.2
Digestion of Oak embryogenic suspension cultures in different concentrations of pectinase. Plate shows Oak cells following a 9 h digestion in 0 gdm^{-3} (A), 0.01 gdm^{-3} (B), 0.03 gdm^{-3} (C), 0 gdm^{-3} (D), 0.07 gdm^{-3} (E) and 0.1 gdm^{-3} (F) pectinase in MS medium. Images were obtained using the OPTIMAS 6 image analysis programme under 10 X magnification. Bar = 2 mm (A), 150 μm^3 (B, C, D), 60 μm^3 (E, F).

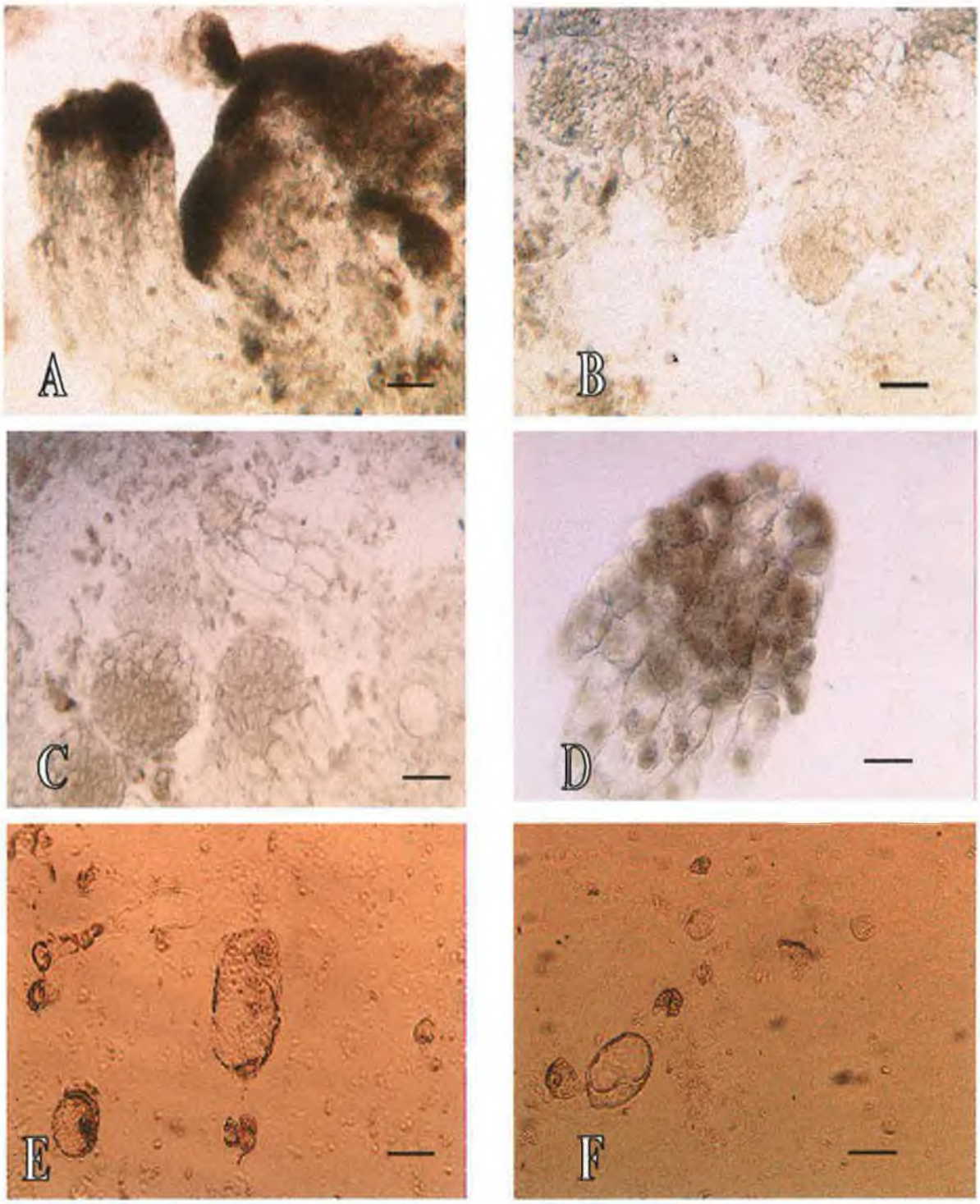


Plate 6.3 Digestion of Sitka Spruce embryogenic suspension cultures in different concentrations of pectinase. Plate shows Sitka Spruce cells following a 9 h digestion in 0 gdm^{-3} (A), 0.01 gdm^{-3} (B), 0.03 gdm^{-3}(C), 0.05 gdm^{-3} (D), 0.07 gdm^{-3} (E) and 0.1 gdm^{-3} (F) pectinase in Sitka Spruce embryo initiation medium. Images were obtained using the OPTIMAS 6 image analysis programme under 10 X magnification. Bar = 200 μm (A, B, C, D), 30 μm (E, F).

6.2.4 Pectinase Digestion and Percoll Fractionation of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Embryonal Suspensor Masses

In order to produce cultures which consist solely of embryogenic head single cells, a method for separation of embryogenic head single cells from suspensor cells was investigated. Klimaszewska, 1989 produced protoplast cultures of *Larix* X *eurolepis* using a discontinuous Percoll density gradient.

From the previous experiment a pectinase concentration of 0.07 gdm^{-3} pectinase was identified as the optimum enzyme concentration for the production of single cells. The pectinase solution was prepared as in the previous experiment and 0.5 g of 7d old cells were added. Digestions were vortexed at full speed for 40s and placed on a shaker at 100 r.p.m in the plant culture room for a 9h incubation period. Digestions were then removed and vortexed for a further 40s before spinning for 15 min at 4,000 x *g* in a centrifuge. The enzyme was then decanted and discarded. The cell pellet was resuspended in 1.2 cm^3 of Sitka Spruce embryogenesis initiation medium. An optimum Percoll concentration was obtained in the laboratory by a taught masters student under my direct guidance and supervision and this was then utilized in this experiment. A 90% Percoll stock was pre prepared from which a 19 % Percoll solution was prepared (0.95 cm^{-3} of 90% Percoll stock and 4.05 cm^3 of embryogenesis medium). Prior to fractionation a 100 µl aliquot was taken from the cell suspension and examined under 10 X magnification on a compound microscope. The number of intact embryogenic heads and free suspensors in the sample were recorded. Of the resuspended mixture of heads and suspensor cells 1 cm^3 was added to 5 cm^3 of 19% Percoll and centrifuged at 4,000 x *g* for 40 min for fractionation to occur. The top fraction was removed and discarded and from the pellet a 100 µl sample was taken

for examination of embryogenic head and suspensor cell numbers. All samples were examined under 10X magnification and embryogenic heads and suspensor cells counted using a cell counter. The remainder of the pellet was resuspended in another 5 cm^3 of pectinase mixture and incubated as above for 5–6h. The enzyme was again removed as before and another 100 µl sample taken for examination. The remainder of the cells were transferred to a 24 well plate and their growth monitored. Growth will be discussed in the next section.

The reduction in the number of intact embryogenic heads following fractionation and again following the second digest is evident in Figure 6.2. The frequency of suspensor cells following fractionation decreased from over 600 to less than 200 and again to less than 100 following the second digestion. It was clear that fractionation reduced the suspensor cell content but they were still present. The next section examines how well these single cells grew in 24 well multi-well plates when monitored with the multi-well plate reader.

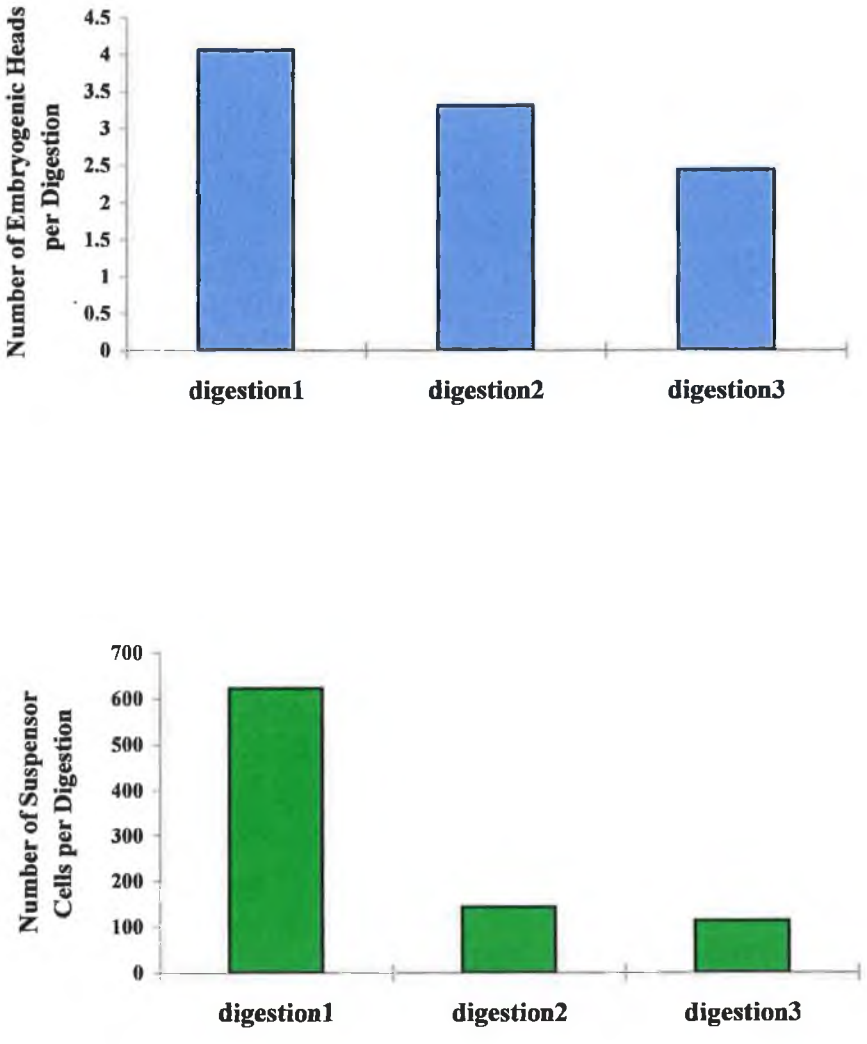


Figure 6.2

Pectinase digestion and Percoll fractionation of Sitka Spruce embryonal suspensor masses. Graphs show the number of embryogenic heads and suspensor cells present after each digestion and fractionation. Samples of 100 µl were observed on the compound microscope under 10 X magnification.

6.2.5 Growth of Sitka Spruce (*Picea sitchensis* (Bong) Carr.) Single Cells in 24 Well Multi-Well Plates

Following pectinase digestion and Percoll fractionation whether or not these embryogenic head single cells would grow in 24 well plates was analysed.

The pellet from the second pectinase digestion was resuspended in 1 cm^3 of Sitka Spruce embryogenesis initiation medium. A number of digests had been carried out and therefore an abundance of cells were available for manipulation. 50% dilutions with fresh Sitka Spruce embryogenesis initiation medium and embryogenic head single cells were prepared up to 1 cm^3 and placed in the wells of the 24 well multi-well plate reader. The 24 well plate was sealed in a single layer of parafilm and inserted into the plate reader in the plant culture room on a shaker at 100 r.p.m. Growth was monitored for 8d.

Cell growth increased from 0d (Fig. 6.3) and was still increasing at 7d. In contrast to growth of embryonal suspensor masses there was no clumping or contamination and it appeared that cells could have remained in the wells for a much longer growth period. The 24 well multi-well plate readers proved suitable for the culture of embryogenic head single cells as they continued to grow up to 7d without contamination or clumping.

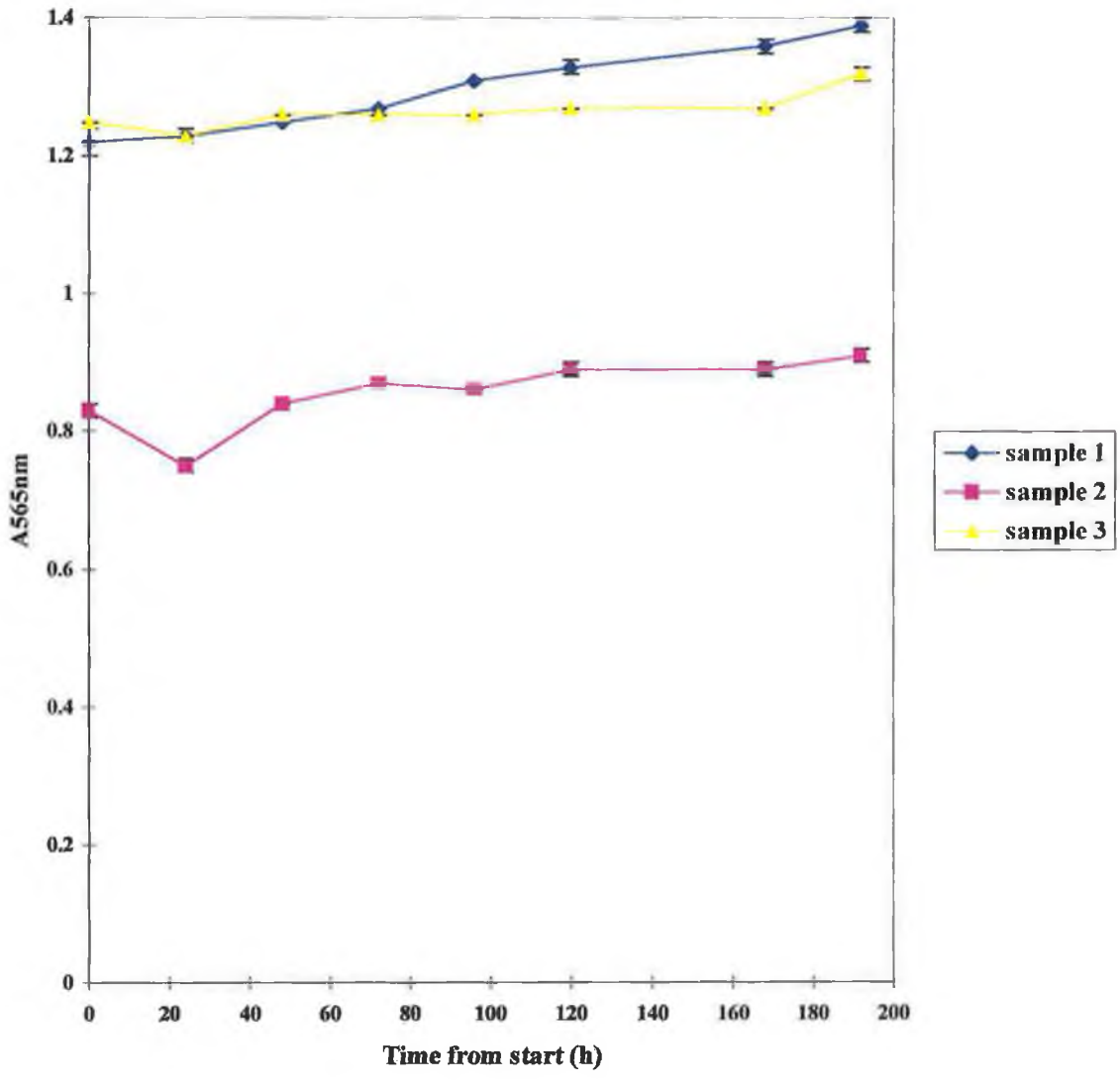


Figure 6.3

Growth of Sitka Spruce single cells in 24 well multi-well plates. Growth was monitored in a 24 well multi-well plate reader in the plant culture room. Results were recorded from 3 wells and experiments were carried out in duplicate and standard error bars included.

6.2.6 Production and Regeneration of Oak (*Quercus robur* L.) Single Cells

Oak single cells can be produced by pectinase digestion. However, pectinase is impure and can lead to complete cell digestion. A method of single cell production other than pectinase digestion was investigated in the experiments below.

Oak embryogenic suspension cultures were transferred to MS medium with a 100-fold reduction in BAP from 1 $mgdm^{-3}$ to 0.01 $mgdm^{-3}$. Cultures were left in the growth room on a shaker at 100 r.p.m for 10d. A 1 cm^3 sample was taken and image analysis performed on the OPTIMAS 6 image analysis programme. The single cells were sieved out with a 500 micron mesh and subcultured into MS medium with an increased BAP concentration of 100 µl. Again cultures were placed in the growth room for a further 10d and a 1 cm^3 sample taken again for image analysis. Cultures were then subcultured onto MS medium with 0.20 $mgdm^{-3}$ of BAP and treated as above. The final subculture was onto MS medium plus 0.3 $mgdm^{-3}$ of BAP. Image analysis samples were analysed for single cell growth and regeneration into callus. Cell samples (100 µl) were taken aseptically every 5d and viability studies carried out with Evans blue (1$mgdm^{-3}$).

Results show that reducing the BAP concentration leads to the dissociation of single cells from larger callus pieces in suspension culture (Plate 6.4). Results also show that increasing the BAP concentration induced the development of cell clusters and micro-calli from these dissociated single cells. Cell viability of dissociated single cells decreased from 0d but levelled off again at 30d to approximately 65% (Fig. 6.4).

Large volumes of single cells were then available for experimentation. The next set of experiments examines the growth of Oak single cells in varying concentrations of cefotaxime. *Agrobacterium*-mediated transformation of these dissocitaed single cells should be possible and the following experiments examine this theory.

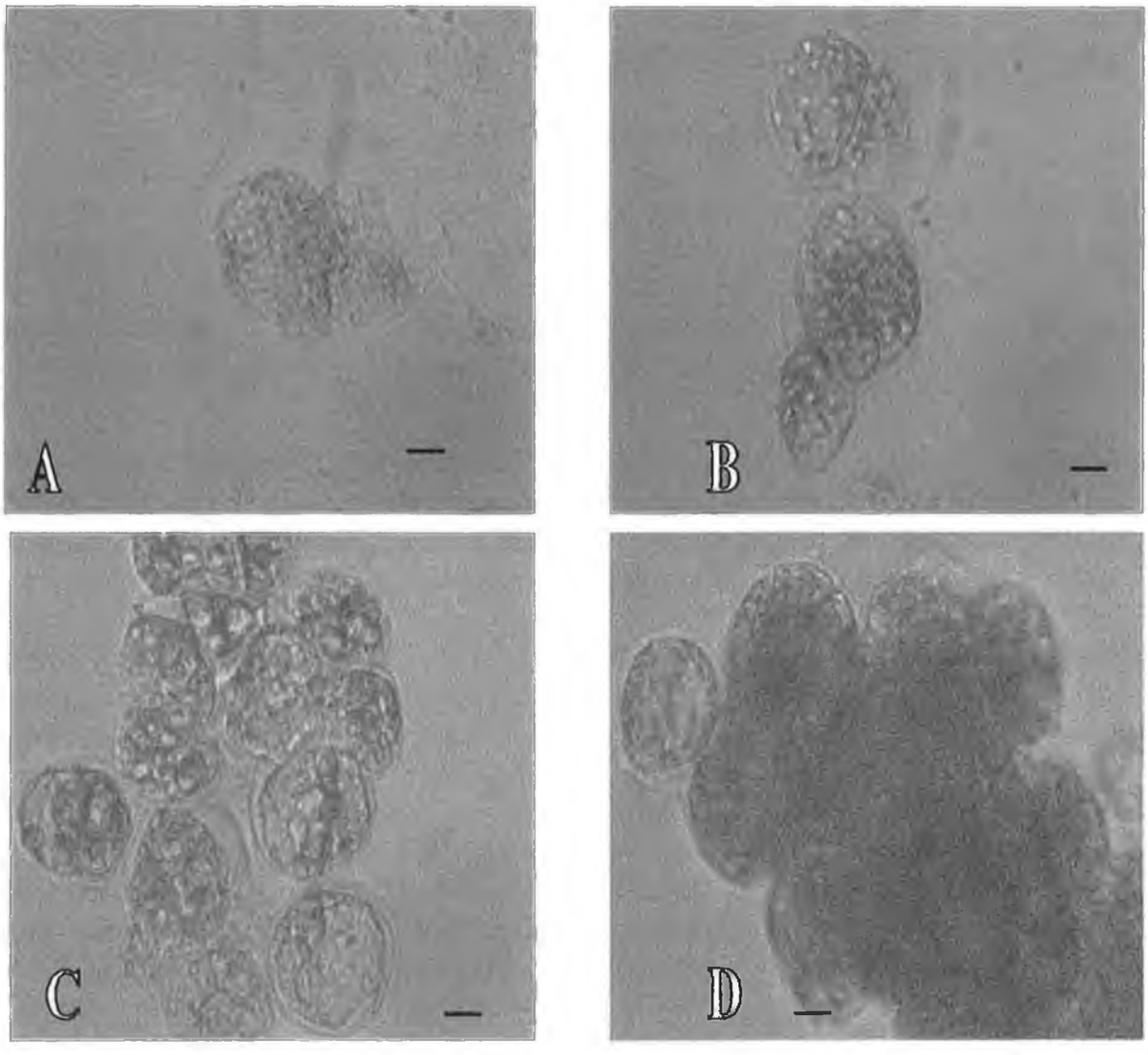


Plate 6.4

Growth of Oak embryogenic cells in increasing concentrations of benzylaminopurine. Plate shows growth of Oak single cells (A) produced from decreasing the BAP concentration from 1 $mgdm^{-3}$ to 0.01 $mgdm^{-3}$ in suspension cultures. Every 10d the concentration was increased from 0.1 $mgdm^{-3}$ (B) to 0.2 $mgdm^{-3}$ (C) and finally at 30 d to 0.3 $mgdm^{-3}$ BAP (D). Images were taken using the OPTIMAS 6 image analysis programme under 40 X magnification. Bar = 15 μm.

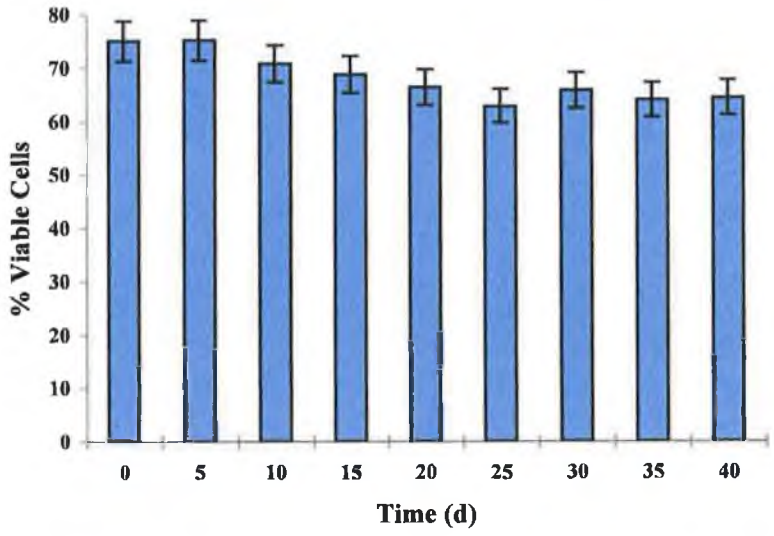


Figure 6.4

Cell viability (%) of dissociated Oak single cell cultures. 100 µl samples were stained with Evans blue and examined under 10 X magnification on a compound microscope. Samples were taken in duplicate and standard errors of the means are included.

6.2.7 Growth of Oak (*Quercus robur* L.) Single Cells in Varying Concentrations of Cefotaxime Antibiotic

The growth of dissociated Oak single cells in varying concentrations of cefotaxime antibiotic was assessed with a view to transforming single cells with *Agrobacterium*.

The contents of five 7d old liquid cultures of Oak single cells were dispensed into 20 cm^3 sterile Universal bottles in 6 cm^3 aliquots and spun down at 3,500 x *g* for 10min. The supernatant was removed and cells were resuspended in 6 cm^3 of MS in different concentrations of cefotaxime antibiotic 0 $mgdm^{-3}$, 500 $mgdm^{-3}$, 1000 $mgdm^{-3}$ and 2000 $mgdm^{-3}$. In a 24 well plate, the medium plus cefotaxime plus Oak cells were dispensed in 1 cm^3 aliquots per well filling 6 wells per antibiotic concentration. Plates were sealed with a single layer of parafilm and placed on a gyratory shaker at 100 r.p.m in the plant culture room. Cell growth readings were taken three times a day for 7d.

Results show that as was previously found with Oak embryogenic suspension cultures, concentrations cefotaxime above 500 $mgdm^{-3}$ hinder cell growth (Fig. 6.5). Growth at 1000 $mgdm^{-3}$ and 2000 $mgdm^{-3}$ cefotaxime concentration resulted in a decrease in cell growth after 1d.

Cefotaxime was proven to be suitable for the elimination of *Agrobacterium* at concentrations of 500 $mgdm^{-3}$ and below. These results lead the way for the transformation of single cells and this was investigated in the following experiment.

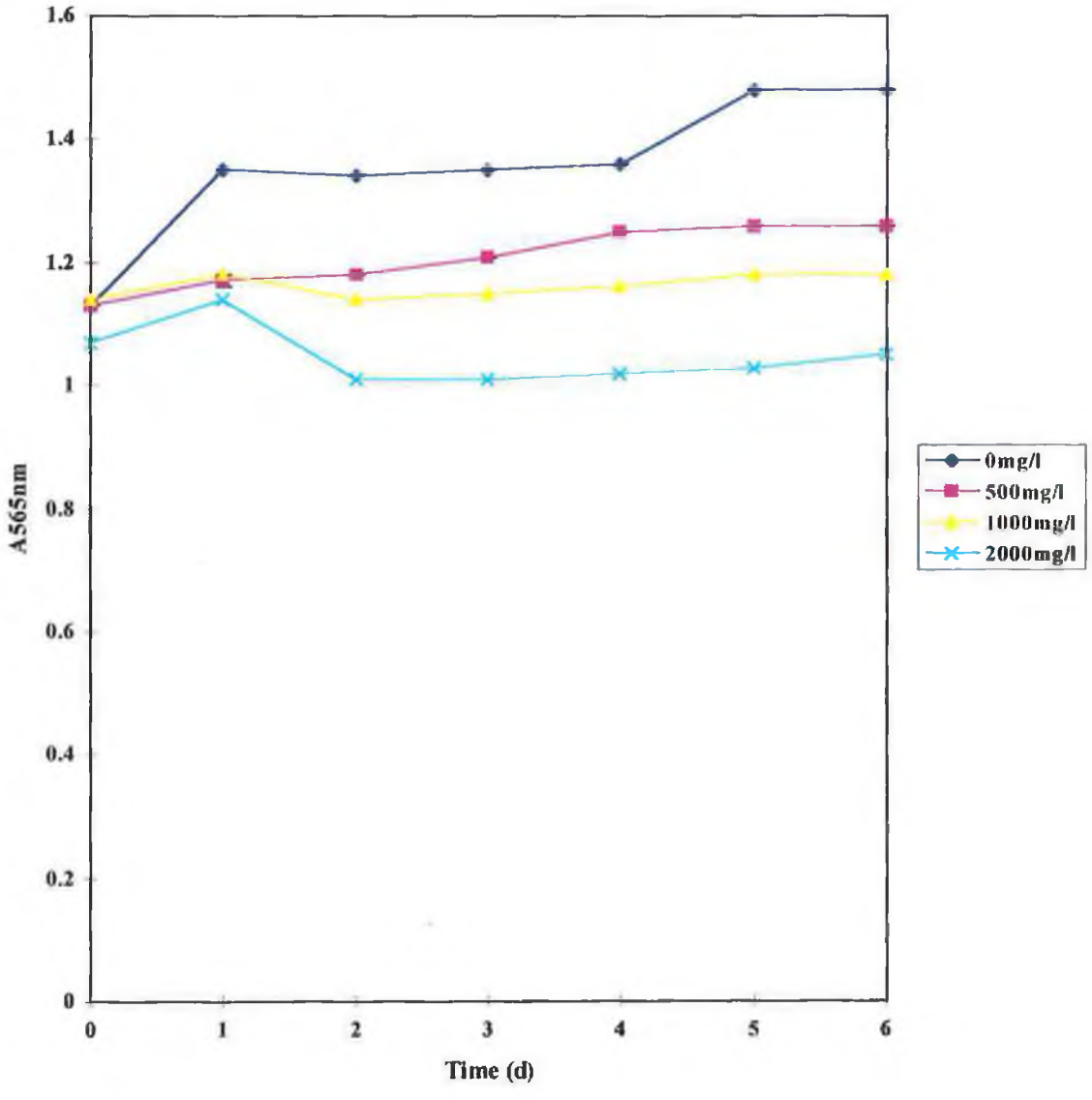


Figure 6.5

Growth of Oak (*Quercus robur* L.) single cell suspensions in different concentrations of cefotaxime antibiotic (0 $mgdm^{-3}$ - 2000 $mgdm^{-3}$). Cell growth was monitored in a 24 well multi-well plate reader in the plant culture room. Results were taken from the average of 6 wells per treatment. The experiment was carried out in duplicate and standard error bars ae included.

6.2.8 *Agrobacterium*-mediated Transformation of Oak (*Quercus robur* L.) Single Cell Cultures

Expression of *GUS* A gene in Oak single cell cultures was investigated in the following experiment.

From 7d old Oak single cell suspensions, 5 cm^3 aliquots were dispensed into sterile 20 cm^3 Universal bottles. Samples were spun down for 15min at 3,500 x *g* and the medium removed and discarded. Cells were resuspended in a 50% dilution of an overnight culture of *Agrobacterium* LBA4404::pBI121 plus 25 µM acetosyringone in MS medium to 5 cm^3. Cells were incubated in the bacterial suspension for 60min on a shaker at 100 r.p.m in the plant culture room. Cells were then spun again for a further 15min at 3,500 g and the supernatant removed and discarded. The cells were washed twice in sterile distilled water and finally resuspended in 5 cm^3 of MS minus antibiotics and co-cultivated with the residual *Agrobacterium* for 48h on a shaker at 100 r.p.m in the plant culture room. Following co-cultivation, cells were again spun to remove the medium and resuspended in 5 cm^3 of MS plus 500 $mgdm^{-3}$ cefotaxime antibiotic to inhibit further growth of *Agrobacterium*. Every 3d a 100 µl sample was taken and a histochemical assay performed to test for the continued expression of the *GUS* A gene. The cells were subcultured as normal every 10d. Control transformations were carried out in the same manner but with *Agrobacterium* LBA4404 which did not contain the plasmid.

GUS A expressing Oak single cells can be seen in Plate 6.5. The blue colouration confirms expression of the *GUS* gene. Cell numbers were not recorded as this was simply an experiment to investigate whether or not transformation would occur. Control transformations failed to stain blue.

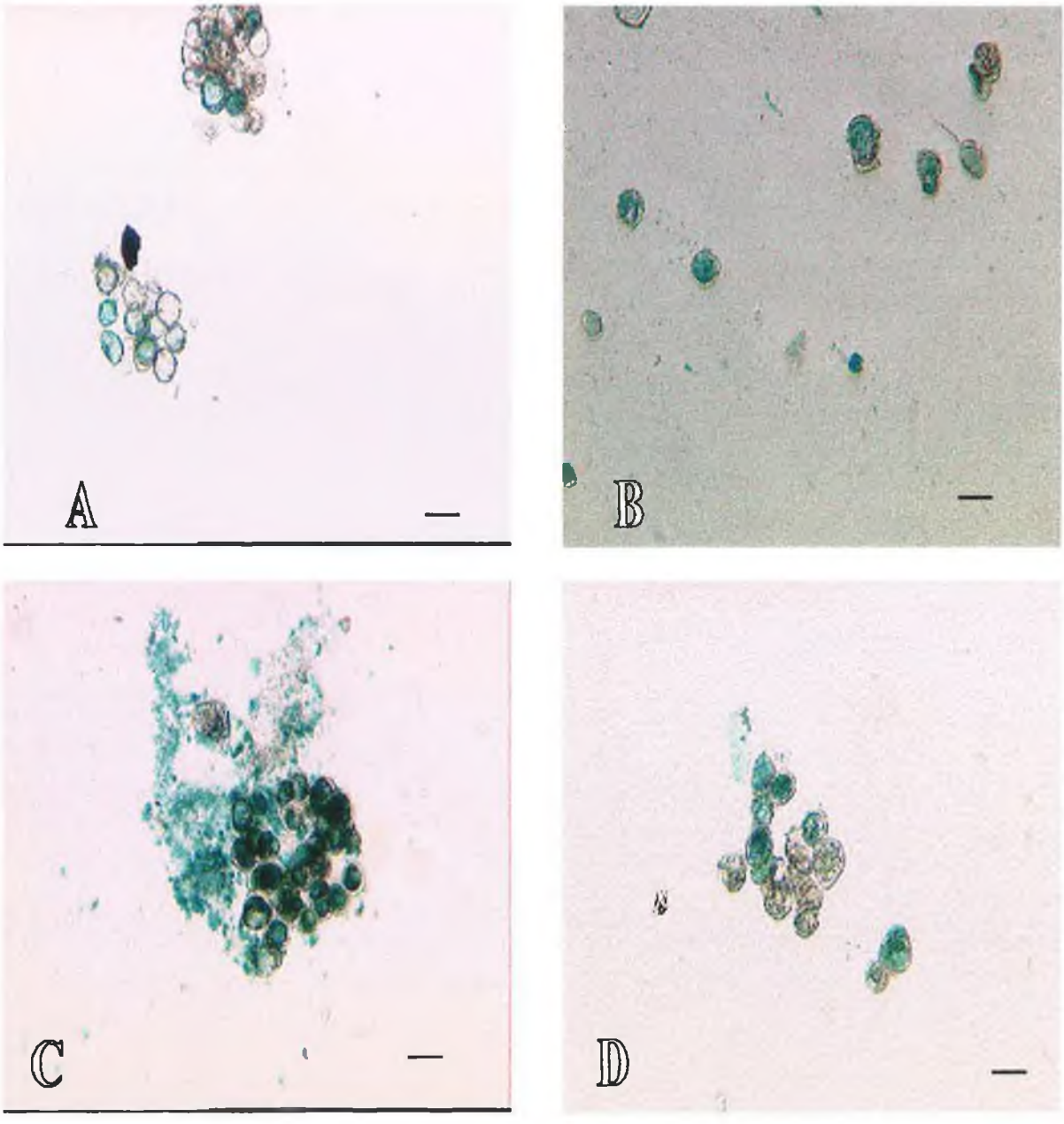


Plate 6.5

Oak single cells and cell aggregates expressing *GUS* A gene at 5 d (A), 10 d (B), 20 d (C) and 30 d (D) after inoculation with *Agrobacterium* dilutions. Bar = 60 μm^3. Debris seen around cells (C) were single cells which burst and released their contents to the environment.

6.3 Discussion

The main aims of producing single cells from embryogenic suspension cultures of Oak (*Quercus robur* L.) and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) were both to study regeneration ability and to eventually transform cells and produce a whole transformed plantlet.

The production of a 24 well multi-well plate reader to monitor cell growth in 24 well multi-well plates proved extremely fruitful for monitoring single cell growth in particular. Previous reports on protoplast culture of woody species have cultured protoplasts in petri dishes ranging in size from 60mm–3.5 cm in diameter (Wang *et al.*, 1995; Brison *et al.*, 1990). The use of this 24 well multi-well plate reader allowed for non-invasive monitoring of cell growth, in contrast to petri dish culture which needed to be sampled and thus introducing the element of possible contamination.

An important factor in single cell isolation is to establish rapidly growing cell suspensions of donor species. Oak and Sitka Spruce embryogenic suspension cultures at 7d after subculture were used as a source of cells in this experiment. In the majority of studies on protoplast culture, suspension cultures ranging from 3d–7d after subculture were used. Fowke *et al.*, 1995 reported that embryogenic suspensions of spruce consisting of immature embryos are excellent source of regenerable protoplasts.

Methods for isolation of protoplasts vary considerably in published reports. All reports included an enzyme digestion step. The optimum pectinase concentration for producing Sitka Spruce single cells was recorded at 0.07 gdm^{-3} for 9h and a second digestion for 5–6h on a gyratory shaker at 100 r.p.m. A combination of cellulase and macerozyme digestion was utilised in most reports of protoplast production (Wang *et al.*, 1995; Qiao *et al.*, 1998; Klimaszewska, 1989; Brison *et al.*, 1990). Digestion times ranged

from 1.5h–6h. All digestions were incubated in plant culture rooms on a gyratory shaker at speeds ranging from 16 r.p.m–40 r.p.m. Single cell cultures of Oak were produced by incubating embryogenic suspension cultures in 0.07 gdm^{-3} pectinase for 9h. However, as a cheaper and more efficient alternative single cell cultures of Oak could be easily produced by reducing the concentration of benzylaminopurine in the medium. Embryogenic head single cells of Sitka Spruce were separated from single suspensor cells and cell debris by a 19% Percoll density gradient. Pellets formed contained embryogenic head single cells whereas the middle and top fractions contained cell debris and suspensor cells. A discontinuous Percoll density gradient has been used for protoplast fractionation of *Larix X eurolepis* (Klimaszewska, 1989). Ficoll density gradient separation has been performed for the production of root protoplasts of *Q. rubra* L. (Brison *et al.*, 1990).

Regeneration of whole plants from protoplast cultures has been reported for *Larix X eurolepis* (Klimaszewska, 1989); *Populus alba* L. (Qiao *et al.*, 1998); *Simon populus* (Wang *et al.*, 1995). Brison *et al.*, 1990, reported callus regeneration from root protoplasts of *Quercus rubra* L. Protoplasts from embryogenic material can be regenerated to embryos and consequently to plants in *Q. petraea* (Jorgenson, 1993). Studies carried out on *Picea abies* by Egertsdotter and von Arnold, 1993 revealed that even with this model conifer species problems with the regeneration of protoplasts were experienced. Protoplasts derived from embryogenic cultures which grew well in suspension and exhibited an embryogenic head of loosely packed cells went on to enter the embryogenic pathway and continued to grow to give rise to somatic embryos. However it was noted that fewer protoplasts from these suspension cultures entered the embryogenic pathway than did protoplasts derived from embryogenic cultures which grew well on solid media and exhibited an embryogenic head region of densely packed cells. These protoplasts however, did not continue to grow and give rise to somatic embryos. Increasing the BAP

concentration in Oak (*Quercus robur* L.) single cell cultures resulted in the formation of large cell aggregates and micro-calli after 40d in culture. Studies on the regeneration of Sitka Spruce protoplasts were not carried out. However it has been reported that for protoplast cultures of Loblolly pine and Douglas fir, the colonies of regenerated embryogenic tissue originated from small highly cytoplasmic protoplasts rather than larger vacuolated suspensor protoplasts (Gupta and Durzan, 1987; Gupta *et al.*, 1988). Behrendt and Zoglauer, 1996 reported that protoplasts prepared from an embryogenic suspension culture of *Larix decidua* Mill. required high levels of boric acid (100 mu M) in order for the compact colonies formed to produce polar suspensors.

Successful transformation of protoplast cultures has been reported for a number of plant species, *Triticum-aestivium* L. (Zaghmont , 1994); *Oryza-sativa* (Biswas *et al.*, 1994); Poplar *spp.* (Chupeau *et al.*, 1994); *Glycine max* [L.] Merr. (Dhir *et al.*, 1992). All transformations however were carried out by electroporation or polyethylene glycol with the exception of wheat which was transformed by *Agrobacterium*-mediated transformation. Transient *Agrobacterium*-mediated transformation of Oak (*Quercus robur* L.) single cells was confirmed by the presence of blue stained cells following a histochemical *GUS* assay. The ability of single cells to continuously grow in medium containing concentrations of cefotaxime antibiotic as high as 500 $mgdm^{-3}$ allowed for the use of cefotaxime in the elimination of *Agrobacterium* after the co-cultivation period.

6.4 Conclusion

Suspension cultures of single cells of Oak (*Quercus robur* L.) and Sitka Spruce (*Picea sitchensis* (Bong) Carr.) were produced from 7d old embryogenic suspension cultures. The growth of single cell cultures was continuously monitored with the use of a 24 well multi-well plate reader constructed in the laboratory. Growth of Oak single cells was monitored in MS medium containing cefotaxime antibiotic. Oak single cells were capable of growing in concentrations of cefotaxime as high as 500 $mgdm^{-3}$. Oak single cells were regenerated to micro-calli by increasing the BAP concentration in the medium. Oak single cells expressing the *GUS* A gene were achieved using the *Agrobacterium tumefaciens* strain LBA4404::pBI121 containing the *gus* and *npt* II genes. These results lead the way to the production of whole Oak plantlets expressing the *GUS* A gene.

Optimized Protocol for the Production of Oak Single Cell Cultures From Embryogenic Suspension Cultures

Preparation of 1 dm^3 MS medium (200 $mgdm^{-3}$ L-glutamine, 0.01 $mgdm^{-3}$ BAP)

$50cm^3$ dispensed into sterile 250 cm^3 Erlenmeyer flasks

Oak embryogenic suspension cultures sieved through 100 μm mesh and transferred to 250 cm^3 Erlenmeyer flasks plus 50 cm^3 MS (200 $mgdm^{-3}$ L-glutamine, 0.01 $mgdm^{-3}$ BAP.)

growth room, 100 rpm, 10d

Cells sieved (100 μm mesh) and cell aggregates removed and discarded
Single cells and small cell aggregates subcultured into fresh MS medium (200 $mgdm^{-3}$ L-glutamine, 0.01 $mgdm^{-3}$ BAP.)

Single cells subcultured as normal every 10d into fresh MS medium (200 $mgdm^{-3}$ L-glutamine, 1 $mgcm^{-3}$ BAP.)

To reform cell aggregates from Oak single cell cultures increase the BAP concentration in the MS medium to 1 $mgdm^{-3}$ and subculture every 10d into fresh MS (200 $mgdm^{-3}$ L-glutamine,1 $mgdm^{-3}$ B.A.P.)

Optimized Protocol for the Production of Sitka Spruce Single Cell Cultures From Embryonal Suspensor Masses

Sieve (100 µm mesh) 7d old cultures of Sitka Spruce

↓

Pectinase (0.07gdm^{-3}) dissolved in 5 cm^3 Sitka Spruce embryogenesis, embryo medium and filter sterilize into sterile 20 cm^3 plastic universal

↓

0.5 g ESM added to pectinase solution

↓ vortex 40s, 100 rpm, 9h, growth room

Cells vortexed 40s and centrifuged to pellet cells

↓ 15 min, 4,000 g

Supernatant removed and discarded

Pellet resuspended in 1.2 cm^3 Sitka Spruce embryogenesis, embryo initiation

↓

1 cm^3 sample removed and added to 5 cm^3 19% Percoll solution in 20cm^3 sterile plastic Universal, Sample centrifuged to fractionate

↓ 40 min, 4,000 g

Remove top fraction and discard

↓

Resuspend pellet in 5 cm^3 pectinase solution

↓ vortex 40s, 100 rpm, 5-6h, growth room

Vortex 40s and centrifuge to pellet cells

↓

Discard supernatant and resuspend cells in 5 cm^3 Sitka Spruce embryogenesis, initiation medium

↓

In a sterile 24 well multi-well plate add 0.5 cm^3 of Sitka Spruce embryogenesis, initiation medium plus 0.5 cm^3 of cells from sample to give a 50% cell dilution. Place on shaker in growth room

Chapter 7

General Discussion

7.1 General Discussion

The ability to develop somatic embryo cultures from a number of softwood (Hakman and Von Arnold, 1985; Gupta and Durzan, 1987a; Durzan and Gupta, 1987; Hakman and Fowke, 1987a; Krogstrup *et al.*,1988; Boulay *et al.*,1988) and hardwood (Chalupa, 1985b; El Maatoui and Espagnac, 1987; Gingus and Lineberger, 1989; Feraud-Keller and Espagnac, 1989; Manzanera *et al.*,1992) species, has led to increased experimentation into the production of transgenic trees. The application of classical breeding techniques to tree species has been limited by their long regeneration times. Genetic engineering of somatic embryo cultures is potentially useful in forestry as specific genetic changes could be made in a relatively short period of time. However, the major limitation of genetic engineering of somatic embryos is the low frequency of somatic embryo conversion to plantlets.

Agrobacterium is capable of infecting a number of conifer (Sederoff *et al.*,1986; Ellis *et al.*,1989; Loopstra *et al.*,1990; Stomp *et al.*,1990; Bergmann and Stomp, 1992; Confalonieri *et al.*,1994; Tzfira *et al.*,1996) and *Quercus* species (Evers *et al.*,1988; Roest *et al.*,1991) and was therefore the transformation method of choice for our experiments. *Agrobacterium*-mediated transformation is inexpensive in comparison with biolistic methods of transformation. Before commencing any transformation procedures with *Agrobacterium* a number of preliminary experiments were carried out. Most importantly, a method of eliminating *Agrobacterium* from the cultures following transformation was examined. Results showed that concentrations of cefotaxime as high as 1000 $mgdm^{-3}$ allowed for the continued growth of both Oak and Sitka Spruce embryogenic suspension cultures. Cultures required the continued presence of cefotaxime for at least a further 5-6 subcultures to ensure complete elimination of the bacteria. Levels of cefotaxime must

remain as high as 500 $mgdm^{-3}$ for the duration of all subcultures as it is capable of breaking down in the presence of light. Studies suggested the use of timentin as an alternative to cefotaxime (Naubery *et al.*,1997; Cheng *et al.*,1998). Lower levels of timentin (150 $mgdm^{-3}$) were required to eliminate *Agrobacterium* from transformed tobacco plants. The effects of timentin on the growth of Oak and Sitka Spruce cultures would however need to be investigated. Experiments on the maturation of Sitka Spruce ESMs showed that although cefotaxime had an effect on the number of embryos produced (Figure 5.25) in embryo development medium, the subsequent germination of these embryos into plantlets was not significantly different (Fig. 5.26) to results produced in the absence of cefotaxime. Sufficiently large numbers of embryos were still produced and therefore a reduction due to the presence of cefotaxime was not a major concern.

The selectable marker NPT II which confers resistance to a number of amino glycoside antibiotics (Herrrera-Estrella *et al.*,1988) has been routinely used in a number of transformations for the selection of transgenic plant material (Loopstra *et al.*,1990; Roest *et al.*,1991; Bommineni *et al.*,1993, 1994; Shin *et al.*,1994; Howe *et al.*,1994; Charest *et al.*,1996; Drake *et al.*,1997; Gonzalez *et al.*,1998). Kanamycin was shown to have no inhibitory effect on the growth of the cultures (Fig. 3.5, 3.13) in fact, levels as high as 700 $mgdm^{-3}$ seemed to reduce the doubling time of the Sitka cultures (Fig. 3.6, 3.14). However, Drake *et al.*,1997 while working on *Picea sitchensis* ESMs on solid medium reported inhibition of growth of cultures at 5 $mgdm^{-3}$. A possible explanation for this large difference may be that Drake and his co workers were working with a different clone of Sitka Spruce (IL-1, IL-2, IL-3) which had been maintained on Selby MPM medium with 10μM 2,4-D (John *et al.*,1995). Culture in suspension medium leads to a relatively homogenous and finely dispersed culture at a relatively fast rate. Drake *et al*, results may have been more understandable if they had cultured their Sitka in liquid rather than on solid

medium where only certain areas of the culture may have been in direct contact with the medium. Paromomycin as an antibiotic proved strongly inhibitory to our Oak and Sitka Spruce (30 $mgdm^{-3}$, 3 $mgdm^{-3}$) embryogenic suspension cultures in the short term growth studies. It was obvious however in cultures of Oak which had been expressing β-glucuronidase activity for up to 1 year (Plate 4.1) that some cells had escaped the effects of paromomycin and were present between large sections of β-glucuronidase expressing tissue. Park *et al.*, 1998 examined two selectable markers in the transformation of tobacco, phosphinotricin acetyl transferase (PAT) and hygromycin phosphotransferase (HPT). Experiments were carried out to determine the lethal level of the herbicide glufosinate-ammonium (phosphinotricin) (PPT) using a leaf disc regeneration assay established that no shoots regenerated at 2-4mg PPT per 1. With the antibiotic hygromycin no plants regenerated at 50 mg per 1. In contrast after co-cultivation of the leaf discs with *Agrobacterium tumefaciens* containing either PAT or HPT gene in combination with a *Bt* gene for insect resistance plants were successfully regenerated from leaf discs at 2-4 mg per 1 PPT and 50 mg hygromycin per 1. However plants regenerated at 2 and 3 mg PPT per 1 were found to be non-transformed. This study showed a significant level of cross protection and/or transient expression of the PAT selectable marker gene allowing 95-100% escapes at selection levels of 2-3 mg PPT per 1 which completely kill controls. On the other hand, the HPT gene at 50 mg is efficient in selecting for T-DNA integration. *Agrobacterium*-medated transformation of sugar pine (Loopstra *et al.*, 1990) revealed that following transformation with the NPT II gene as a selectable marker not all callus lines expressed NPT II activity. It was also reported that not all NPT II positive callus lines had the same level of activity. Some lines also survived selection but had no detectable NPT II activity. Stable transformation of black spruce was reported by Charest *et al.*, 1996. Cotyledonary somatic embryos and suspensions from embryonal masses were transformed

via particle bombardment. Charest reported a high percentage of tissues escaped kanamycin selection without being genetically transformed. With a low frequency of transformed tissue recovery it was questionable if kanamycin selection had any effect. However kanamycin toxicity data indicated that there was a proportion of cells that were killed and that selection was occurring. Liquid selection appeared more effective in eliminating non-transformed or non-expressing tissues.

The use of β-glucuronidase as our reporter gene proved highly successful and assays were easily performed. The major disadvantage however was the destruction of valuable transformed material at regular intervals (3-5d) to carry out histochemical assays. The green fluorescent protein (GFP) is proving to be very useful as a reporter gene because it requires no additional substrate to assay merely excitation with near ultra violet (U.V.) or blue light. The advantage of GFP is that assays can be carried out in a non-destructive manner and therefore continued presence of the gene can be monitored without destructively harvesting valuable material. The fluorescence of GFP is very stable and easily visible under normal room fluorescent lighting (Chalfie *et al.*,1994). Eu *et al.*,1998 reported on the use of the chlorophyll florescence assay for kanamycin resistance screening in transgenic plants. Chlorophyll fluorescence has been widely used as a quantitative, sensitive and non-destructive technique to screen photosynthetic mutants (Bennoun and Levine, 1967; Yorvan *et al.*,1983) and to evaluate plant disease progression (Peterson and Aylor, 1995). It should be possible to apply the chlorophyll fluorescence assay to the screening of transgenic plants carrying selectable marker genes which detoxify amino-glycosides, since these antibiotics inhibit protein synthesis by binding at several sites involved in the translation process. Eu *et al.*,1998 using this chlorophyll fluorescence assay was able to score kanamycin sensitive tobacco plants which had been transformed with the NPT II gene via *Agrobacterium*. This demonstrates its use as a reliable and efficient non-

destructive assay for kanamycin resistance screening of transgenic plants. The assay can be used to screen any plant with green tissues showing resistance to a chemical. It may be advantageous to study the use of this assay with paromomycin as a non-destructive method of assaying Oak and Sitka Spruce cultures. However, it would only be of use for assaying maturing cultures at the later stages of development due to the absence of a green pigment in the early stages of embryogenic cultures.

Workable transformation protocols for transient transformation of Oak and Sitka Spruce embryogenic suspension cultures were developed. Results for the transformation of Sitka Spruce ESMs in liquid culture differed from those reported by Drake *et al.,* 1997 with Sitka Spruce ESMs on solid medium. Incubation times and acetosyringone concentrations differed. However, numbers of *GUS* positive loci per gram of cells were significantly lower than those recorded in our experiments (Table 4.14). These variations could be explained by the fact that the cell lines used were different and the fact that our experiments utilized ESMs in liquid culture whereas Drake *et al.,* utilized ESMs on solid medium. Humara *et al.,* 1998 reported on the significance of introns to enhance gene expression. Drake *et al.,* used a CaMV35-*gus*-intron gene in their transformations. Humara experienced an increase in gene expression in pine species by the addition of the intron *Shrunken-1*. In the pine species tested, the *Sh1*-int1 increased transient *GUS* expression from 2 to 6-fold compared to the intron-less construction. This construct also eliminates *Bacterial* expression. The construct used in our experiments did not contain an intron and this should therefore be considered when designing future experiments to achieve stable transformation.

Gus expression was present in transformed Sitka Spruce (Plate 4.2.) cultures for up to 4-5 months and up to 1 year for transformed Oak embryogenic cultures (Plate 4.1). Reasons for transgene loss are unknown but a number of hypotheses have been developed.

Several factors relating to integration and structure of the transgene DNA such as number of transgenic copies, position in the genome, and methylation, may greatly influence expression of transgenes. Transgene silencing is the inactivation of transgene expression despite the presence of an unchanged but possibly methylated transgene sequence in the plant genome. Transgene silencing is associated with specific changes in the transgene integration pattern. The presence of multiple copies at the integration locus was reported to be a causative factor in transgene silencing (Matzke and Matzke, 1997). This phenomenon of transgene silencing is the most plausible reason for the loss of transgenes in our transformed embryogenic cultures. In the optimization of our transformation protocols it is possible that we incubated the cultures with too large quantities of *Agrobacterium* resulting in the integration of multiple copies of our transgenes. Although single copy transgenes can be inactivated (Meyer *et al.,* 1992) transgene inactivation generally occurs at highest frequencies when multiple copies of the gene are integrated either in a single insertion site or when dispersed throughout the genome (Finnegan *et al.,* 1994). Transgene silencing in rice was recently reported (Chareonpornwattana *et al.,* 1999) and was thought to be due to multiple copies of the gene at the integration locus.

A number of methods for improving transgene stability have been suggested; 1). the selection of transgenic plants which contain a single intact copy of the transgene 2). inclusion of nuclear scaffold or a matrix attachment region flanking a transgene can increase the level and reduce the variability associated with transgene expression in plants 3). the use of enhancers from tissue specific or developmentally regulated genes may ensure the expression of linked transgenes occurs in the appropriately regulated manner (Pawlowski *et al.,* 1997).

Transformed Oak embryogenic cultures expressed β-glucuronidase activity up to 1 year in culture. Southern blot analysis was not carried out due to the lack of transformed

tissue. Results showed that the highest number of *GUS* positive loci identified per gram of transformed tissue was 627 (Table 4.7). Taking into account that each cell is approximately 60 μm^3 cell size this produces a transformation efficiency of approximately 0.0063%. It was estimated that to carry out Southern hybridization analysis, at least 10 g of β-glucuronidase expressing tissue would be required. To produce 1 g of transformed tissue up to 20 cell doublings are required and this alone would take up to 200d in culture. As mentioned continuous assaying of transformed tissue to confirm continued β-glucuronidase activity leads to the destructive harvesting of a large quantity of transformed tissue. Repetitive sampling also led to contamination due to continuous disruption of cultures. We feel however that Oak embryogenic cultures assayed 1 year after transformation which exhibited evidence of large *GUS* expressing sectors were indeed stably transformed although there is no concrete evidence to prove this. Southern blots would have been the best test for stable integration of these genes. Blue staining may have been due to residual *Agrobacterium* in the culture medium however, if this was the case the culture medium would have also stained blue but it remained colourless. If *Agrobacterium* were still present, it would tend to be present throughout the cultures instead of specific locations within the cells.

It is obvious therefore that experiments to increase transformation efficiency must be investigated to bulk up stocks of transformed material. Increased amounts of transformed tissue will allow for Southern hybridization analysis to be carried out. More importantly a non-destructive method of assaying transgene expression is required to reduce the loss of very valuable transgenic material.

Somatic embryogenesis is regarded as the *in vitro* system of choice with potential for eventual mass propagation of superior and genetically engineered forest tree genotypes in both coniferous and hardwood species (Gupta *et al.*, 1991). Limitations to

using this technology for mass propagation of elite tree species are the inability to initiate embryogenic callus from non-embryogenic tissue, low frequency of embryo formation, low germination rate, inability to control aberrant morphology and difficulty in acclimatising germinated plantlets to *ex vitro* environments.

Successful maturation and regeneration protocols are in place for *Picea sitchensis* embryogenic cultures. Plantlets of Sitka Spruce have been successfully regenerated and grown in soil (Krogstrup, 1988). However, this is not the case for *Quercus robur* L. embryogenic cultures. Somatic embryogenesis has been reported for a number of *Quercus* species but plant regeneration from Oak somatic embryos has proven difficult. The conversion of somatic embryos into plantlets has been achieved only in some species (*Q. rubra*, Gingas and Lineberger, 1989; *Q. robur*, Chalupa, 1990,1995, Cuenca *et al.*, 1998, Tsvetkov, 1998; *Q. acutissima*, Kim *et al.*, 1994; *Q. petraea*, Chalupa, 1995). Initial source of explant, genotype, nutrient medium and phytohormones greatly influence the induction of embryogenic cultures and plant regeneration.

A variety of treatments including media type, desiccation, addition of exogenous hormones and changes in environmental conditions were utilized in attempts to mature and regenerate Oak embryogenic cultures. Minimal success was achieved on MS and WPM media where callus greening, cotyledon formation and some rooting occurred. However, P24 medium and P24 maturation media yielded both root and shoot proliferation but plantlets could not be recovered.

Desiccation treatment with high osmoticum although previously reported as increasing the germination and production of shoot and roots of *Q. robur* and *Q. petrea* (Chalupa, 1990, 1995) embryogenic cultures proved unsuccessful in our studies. Some success was achieved by raising the agar concentration from 0.6-0.8% in P24 maturation

media. The frequency of roots and shoot proliferation was increased to 15-40% and 15-100% respectively when transferred from P24 medium plus 0.9 μM BAP in the light.

Previous investigations into *Quercus* embryo conversion suggested that the inclusion of exogenous ABA in the media was beneficial to root and shoot proliferation for *Q. robur* embryo cultures (Ostrolucka *et al.*, 1996; Cuenco *et al.*, 1998). Concentrations of ABA used in our experiments ranged from 0 μM -100 μM and showed no significant effect on culture maturation but in fact may have distressed plants as higher levels of anthocyanin were recorded in the presence of ABA in the culture medium. It is possible that these concentrations were too high as concentrations used by Ostrolucka and Cuenco were 0.2 $mgdm^{-3}$ and 2.7 $mgdm^{-3}$ respectively.

Cytokinins and in particular BAP were an essential ingredient for Oak embryo maturation and germination. Low concentrations (0.2 $mgdm^{-3}$-0.6 $mgdm^{-3}$) promoted greening and further embryoid development in *Q. robur* cultures and in some cultures roots and shoots developed. When cultured in the light on WPM or MS with low BAP concentrations our cultures succeeded in producing embryoids and callus became green in colour however, root and shoot proliferation did not occur. In the majority of cases transfer of cultures from P24 medium plus 0.9 μM BAP to P24 maturation media 1-6 yielded much higher root and shoot proliferation compared to cultures transferred from hormone free P24 medium.

Conditions in the plant culture room most importantly light and dark conditions were very significant. In all cultures transfer from light to dark for the final stages of germination resulted in the absence of callus greening and a reduction in cotyledon formation on WPM and MS media. Cultures maturing on P24 medium when transferred from light to dark showed reductions in root and shoot development.

An optimum protocol was developed for Oak (*Quercus robur* L.) embryo maturation. It appears that conditions required for high frequency embryo conversion vary between *Quercus* species. The reasons for failure to mature *Q. robur* cultures under conditions outlined by Chalupa, 1990, 1995 and Cuenco *et al.*, 1998 in our laboratory are unknown. It is possible that environmental conditions may be playing a role or perhaps these maturation conditions were very highly genotype dependent.

Suspensions containing single cells of Oak and Sitka Spruce were derived from embryogenic suspension cultures. Successful regeneration of Oak single cells to micro-calli stage of development was achieved. In this way, an efficient method for transformation of all cells may be achieved without the complications associated with protoplast isolation and culture. There have been numerous reports on protoplast culture from a number of tree species (Gupta and Durzan, 1987; Gupta *et al.*, 1988; Attree *et al.*, 1987; Klimazewska, 1989; Brison *et al.*, 1990; Wang *et al.*, 995; Qiao *et al.*, 1998). Successful regeneration has been achieved from most species. Protoplast isolation can be a difficult process and in most cases requires a source of suspension cells. Embryogenic suspensions consisting of immature embryos are an excellent source of regenerable protoplasts (Fowke *et al.*, 1995).

Egertsdotter and von Arnold, 1993 produced protoplasts from two cell lines of *Abies alba*. Protoplasts were isolated from cell line A which grew on solid media and exhibited a dense somatic head region, and protoplasts from cell line B which grew well in suspension and exhibited an embryogenic head region of loosely packed cells. The highest proportion of dividing cells entering the embryogenic pathway were obtained from cells belonging to group A. However, only the protoplast derived colonies from cell lines belonging to group B growing as suspensions continued to grow and give rise to many somatic embryos. Attree *et al.*, 1987 while working with white spruce protoplasts reported

that protoplasts were only recovered from a line producing embryos at a low frequency as opposed to a high embryo producing line reported by Bekkaoui *et al.*, 1987.

A successful system for the production of Oak single cells from embryogenic callus in liquid culture was achieved by simply reducing the BAP concentration in MS medium. Single cells could easily be regenerated to micro-calli by increasing the BAP concentration. No media changes were required unlike reports on *Abies alba* by Hartmann *et al.*, 1992, where at least two media changes were required for the production of torpedo shaped somatic embryos from protoplasts isolated from suspension medium. Although studies into regeneration of Sitka Spruce single cells were not carried out in this thesis it has been suggested that (Behrendt and Zoglauer, 1996) that suspensor development in somatic embryos may be dependent on the boron concentration in the protoplast culture. Suspensor development in somatic embryos of *L. decidua* is strongly dependent on boron concentration of the protoplast culture medium. A boron deficiency suppresses suspensor formation. It is possible that boron may play a role in cell wall development based on the assumption that the cell walls of the suspensor differ from the cell walls that make up the embryo proper.

Direct gene transfer using protoplasts has been successfully used to obtain stable transgenic plants (Koop *et al.*, 1996; Dhir *et al.*, 1992; Shimamoto *et al.*, 1989). More recently forest trees have received more attention and in particular *Populus* species have proven to be a suitable model tree. Successful transient *Agrobacterium*-mediated transformation was achieved in Oak single cell cultures in this study. Qiao *et al.*, 1998 in his studies on plantlet regeneration from protoplasts of *Populus alba* L. suggested that *Agrobacterium* gene transfer is more successful in *Agrobacterium* host plants than direct gene transfer. However based on regeneration systems for protoplasts direct gene transfer is simple and reproducible for the production of transgenic plants in any species.

Wheat protoplasts derived from slow growing embryogenic callus showed that *Agrobacterium*-mediated transformation with a binary vector (pKIWI105) succeeded in transforming a higher proportion of cells that electroporation and PEG with the same vector. Soybean protoplasts have also been transformed using *Agrobacterium* but at a low frequency (Baldes *et al.*, 1987).

The advantage of the single cell system for transforming Oak is that instead of large embryogenic aggregates where the *Agrobacterium* may not be capable of reaching and transforming all of the cells, single cell transformation and transformation of small cell aggregates allows all potentially embryogenic and regenerable cells to be transformed. Studies into Sitka Spruce single cell transformation need to be investigated in future experiments but it is reasonable to assume that transient transformation will be achievable based on the results with Oak.

7.2 Future perspectives

Increasing research and success in the area of somatic embryogenesis in woody species has led to their use in a variety of transformation procedures. Transformation of embryogenic cultures eliminates the problems of long regeneration times previously associated with conventional breeding programmes. Transient transformation of Oak and Sitka Spruce embryogenic suspension cultures was achieved in this study. Stable transformation was not conclusively proven. Factors such as the inclusion of introns and most importantly the use of fluorescent selectable markers should be included in future work. Southern blot analysis was not carried out as constant sampling for assays led to a large reduction in the amount of transformed cells and to contamination leading to further

loss. Studies into transgene silencing and inactivation need to be examined in the future to assess reasons for gene loss with time.

The production of single cell cultures for both tree species and the transient transformation and regeneration of Oak single cells to micro-calli opens up a new avenue for investigation into transformation without the problems associated with protoplast culture. Problems with the regeneration of Oak embryogenic cultures have been discussed. An optimized protocol is presented which succeeded in achieving both root and shoot development. Further studies into the effects of cefotaxime on regeneration of Oak need to be examined now that a method of regenerating embryogenic callus has been achieved in the laboratory.

References

Abdul-Baki, A.A., Saunders, J.A., Mathews, B.F. & Pittarelli, G.W. (1990). DNA uptake during electroporation of germinating pollen grains. *Plant Science* 70, 181-190.

Abelson, P.H. & Hines, P.J. (1999). The plant revolution. *Science* **285,** 367-368.

Ahokas, H. (1989). Transfection of germinating barley seed electroporetically with exogenous DNA. *Theoretical and Applied Genetics* 77, 469-472.

Akiyoshi, D.E., Klee, H., Amasino, R.M., Nester, E.W. & Gordon, M.P. (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proceedings of the National Acadamy of Science USA* **81**, 5994-5998.

Albright, L.M., Yanofsky, M.F., Lereux, R.M. & Nester, E.W. (1987). Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear single stranded T-DNA. *Journal of Bacteriology* **169**, 1046-1055.

Ammirato, P.V. (1983). Embryogenesis In: Evans, D.A., Sharp, W.R., Ammarito, P.V. and Yamacta, Y. (eds), *Handbook of plant cell culture*, Vol. 1, *Techniques for propagation and breeding*, 82 - 123 Macmillan Publishing Company, New York.

Attree, S.M., Bekkaoui, F., Dunstan, D.I. & Fowke, L.C. (1987). Regeneration of somatic embryos from protoplasts isolated from an embryogenic suspension culture of White Spruce (*Picea glauca*). *Plant Cell Reports* **6**, 480-483.

Attree, S.M., Tautorus, T.E., Dunstan, D.I. & Fowke, L.C. (1990b). Somatic embryo maturation, germination and soil establishment of plants of Black and White Spruce (*Picea mariana* and *Picea glauca*). *Canadian Journal of Botany* **68,** 2583 – 2589.

Baldes, R., Moss, M. & Geider, K. (1987). Transformation of soybean protoplasts from permanent suspension cultures by cocultivation with cells of *Agrobacterium tumefaciens*. *Plant Molecular Biology* **2**, 135-145.

Barrett, C., Cobb, E., McNicol, R. & Lyon, G. (1997). A risk assessment study of plant genetic transformation using *Agrobacterium* and applications for analysis of transgenic plants. *Plant cell, Tissue and Organ Culture* **47**, 135 – 144.

Barry, G.F., Rogers, S.G., Fraley, R.T. & Brad, L. (1984). Identification of a cloned cytokinin biosynthetic gene. *Proceedings of the National Acadamy of Science USA.* **81**, 4776-4780.

Bayliss, M.W. (1977). Factors affecting the frequency of tetraploid cells in a predominately diploid suspension of *Daucus carota*. *Protoplasma* **92**, 109-115.

Beachy, R.N. (1991). Plant genetic transformation for virus resistance. *Annals of the New York Academy of Science* **646**, 223-227.

Beachy, R.N. (1999). Facing fear of biotechnology. *Science* **285**, 335.

Beaty, J.S., Powell, GK., Lica, L,. Regier, D.A., MacDonald, E.M.S., Hommes, N.G. & Morris, R.O. (1986). *Tzs*, a nopaline Ti plasmid gene from *Agrobacterium tumefaciens* associated with trans-zeatin biosynthesis. *Molecular and General Genetics* **203**, 274-280.

Becwar, M.R., Wann, S.R. & Kriebel, H.B. (1988). In: Hannover, J.W., Keathly, D.E. (eds), *Genetic Manipulation of Woody Plants*, 458 Plenum Press.

Behrendt, U. & Zoglauer, K. (1996). Boron contols suspensor development in embryogenic cultures of *Larix decidua*. *Physiologia Plantarum* **97**, 321–326.

Bekkaoui, F., Saxena, P.K., Attree, S.M., Fowke, L.C. & Dunstan, D.I. (1987). The isolation and culture of protoplasts from an embryogenic cell suspension culture of *Picea glauca* (Moench) Voss. *Plant Cell Reports* **7**, 481-484.

Bekkaoui, F., Pilon, M., Laine, E., Rafu, D.S.S, Crosby, W.L. & Dunstan, D.I. (1988). Transient gene expression in electroporated *Picea glauca* protoplasts. *Plant Cell Reports* **7**, 481-484.

Bekkaoui, F., Datla, R.S.S, Pilon, M., Tautorus, T.E., Crosby, W.I. & Dunstan, D.I. (1990). The effect of promotor on transient expression in conifer cell lines. *Theoretical and Applied Genetics* **79**, 353-359.

Bellarosa, R. (1981). *In vitro* culture of *Quercus suber* L. embryos. In: Colloque In. Cult. In vitro des essen. Forest. AFOCEL, Nangis, 119-126.

Bellarosa, R. (1989). Oak (*Quercus spp.*) In: Y.P.S. Bajaj (ed) Biotechnology in Agriculture and Forestry, Vol. **5**, Tress II, 387-401. Springer-Verlag, Berlin.

Bennoun, P. & Levine, R.P. (1967). Detecting mutants that have impaired photosynthesis by their increased level of flourescence. *Plant Physiolgy* **42**, 1284-1287.

Bergmann, B.A. & Stomp, A.M. (1992). Effect of host plant genotype and growth rate on *Agrobacterium tumefaciens*-mediated gall formation in *Pinus radiata*. *Phytopathology* **82**, 1457-1462.

Bevan, M., Barnes, W. & Chilton, M.D. (1983). Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucleic Acid Research* **11**, 369-385.

Bevan, M.W. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acid Research* **12**, 8711-8721.

Bevan, M.W., Mason, S.E. & Goelet, P. (1985). Expression of Tobacco mosaic virus coat protein by a cauliflower mosaic virus promoter in plants transformed by *Agrobacterium*. *EMBO Journal* **4**, 1912-1926.

Bhagwat, B., Vieira, L.G.E. & Erickson, L.R. (1996). Stimulation of *in vitro* shoot proliferation from nodal explants of Cassava by thidiazuron, benzyladanine and giberellic acid. *Plant Cell, Tissue and Organ Culture* **46**, 1-7.

Bidney, D., Scelonge, C., Martich, J., Burrus, M., Sims, L. & Huffman, G. (1992). Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. *Plant Molecular Biology* **18**, 301-313.

Birt, D.M. (1991). Somatic embryogenesis in conifers. P.hD. Thesis. University of Aberdeen.

Biswas, G.C.G., Iglesias, V.A., Datta, S.K. & Potrykus, I. (1994). Transgenic Indica-rice (*Oryza-sativa*) plants obtained by direct gene-transfer to protoplasts. *Journal of Biotechnology* **32**, 1–10.

Blundy, K.S., Blundy, M.A.C., Carter, D., Wilson, F., Park, W.D. & Burrell, M.M. (1991). The expression of class 1 patatin gene fusions in transgenic Potato varies both with gene and cultivar. *Plant Molecular Biology* **16**, 153-160.

Boerjan, W., Baucher, M., Doorsselaere, J.V., Christiansen, J.H., Meyermans, H., Chen, C., Leple, J.C. & Chognot, E. (1995). Genetic engineering of lignin biosynthesis in Poplar. In: *Somatic Cell Genetics and Molecular Genetics in Trees*. Gent, Belguim.

Bolten, G.W., Nester, E.W. & Gorden, M.P. (1986). Plant phenolic compounds induce expression of *Agrobacterium tumefaciens* loci needed for virulence. *Science* **232**, 983-985.

Bommineni, V.R., Chibbar, R.N., Datla, R.S.S. & Tsang, E.W.T. (1993). Transformation of White spruce (*Picea glauca*) somatic embryos by microprojectile bombardment. *Plant Cell Reports* **13**, 17-23.

Bommineni, V.R., Datla, R.S.S. & Tsang E.W.T. (1994). Expression of gus in somatic embryo cultures of Black spruce after microprojectile bombardment. *Journal of Experimental Botany* **45**, 491-495.

Bonneau, L., Beranger-Novat, N. & Monin, J. (1994). Somatic embryogenesis and plant regeneration in a woody species the European Spindle Tree (*Euonymus europaeus* L.). *Plant Cell Reports* **13**, 135–138.

Boulay, M.P., Gupta, P.K., Krogstrup, P. & DuRzan, D.J. (1988). Development of somatic embryos from cell suspension cultures of Norway spruce (*Picea abies* Karst.). *Plant Cell Reports* **7**, 134–137.

Brandle, J.E., McHugh, S.G., James, L., Labbe, H. & Miki, B.L. (1995). In stability of transgene expression in field grown tobacco carrying the *csr1-1* gene for sulfonylurea herbicide resistance . *Bio/Technology* **13**, 994-998.

Brison, M. & Lamant, A. (1990). Callus formation from root protoplasts of *Quercus rubra* L., (Red oak). *Plant Cell Reports* **9**, 139–142.

Brisson, N., Paszkowski, J., Penswick,J., Gronenborn, B., Potrykus, I. & Hohn, T. (1984). Expression of a bacterial gene in plant cells using a viral vector. *Nature* **310**, 511-514.

Bryant, J. & Leather, S. (1992). Removal of selectable marker genes from transgenic plants: needless sophistication or social necessity? *Trends in Biotechnology* **10**, 274-275.

Bueno, M.A., Astorga, R. & Manzanera, J.A. (1992). Plant regeneration through somatic embryogenesis in *Q. suber*. *Physiological Plant* **85**, 30-34.

Calgene, (1990). Kanr Gene: Safety and use in the production of genetically engineered plants, request for advisory opinion. Calgene Inc. California, USA.

Caplan, A., Herrera-Estrella, L., Inze, D., Van Haute, E., Van Montagu, M., Schell, S. & Zambryskii, P. (1983). Introduction of genetic material into plant cells. *Science*, **222,** 815-821.

Chalfie, M., Yuan, T., Euskirchen, G., Ward, W.W. & Prasher, D.D. (1994). Green flourescent protein as a marker for gene expression. *Science* **263**, 802-805.

Chalupa, V. (1979). *In Vitro* propagation of some broad-leaved forest trees. *Commun. Inst. For. Cech.* **11**, 159-170.

Chalupa, V. (1981). Clonal propagation of broad-leaved forest trees in vitro. *Commun. Inst. For. Cech.* **12,** 255-271.

Chalupa, V. (1985b). *In vitro* propagation of *Larix, Picea, Pinus, Quercus, Fagus* and other species using adenine–type cytokinins and thidiazuron. *Commun. Inst. For. Cech* **14,** 65–90.

Chalupa, V. (1987). Somatic embryogenesis and plant regeneration in *Picea, Quercus, Betula, Tilia, Robinia, Fagus* and *Aesculus. Commun. Inst. For. Cech.* **15**, 133-148.

Chalupa, V. (1988). Large scale micropropogation of *Quercus robur* L. using adenine-type cytokinins and thidiazuron to stimulate shoot proliferation. *Biologia Plantarum* **30**, 414–421.

Chalupa, V. (1990). Plant regeneration by somatic embryogenesis from cultured immature embryos of Oak (*Quercus robur* L.) and Linden (*Tilia cordata* Mill). *Plant Cell Reports* **9**, 398–401.

Chalupa, V. (1993). Vegeatative propagation of Oak (*Quercus robur and Quercus petraea*) by cutting and tissue culture. *Annals of Scientific Forestry* 50, suppl. **1**, 295-307.

Chalupa, V. (1995). Somatic embryogenesis in Oak (*Quercus* spp.). In: Jain, S., Gupta, P. & Newton, R. (eds.), Somatic embryogenesis in Woody Plants. **2**, 67–87. Kluwer Academic Publishers. Printed in the Netherlands.

Chang, W.C. & Hsing, Y.I. (1980). Plant regeneration through somatic embryogenesis in root derived callus of ginseng (*Panax ginseng* C.A. Meyer). *Theoretical and Applied Genetics* **57**, 133–135.

Chareonpornwattana, S., Thara, K.V., Wang, L., Datta, S.K., Panbangred, W. & Muthukrishnan, S. (1999). Inheritance, expression, and silencing of a chitenase transgene in rice. *Theoretical and Applied Genetics* **98**, 371-378.

Charest, P.J, Calero, N., Lachance, D., Datla, R.S.S., Duchesne, L.C. & Tsang, E.W.T. (1993). Microprojectile DNA delivery in conifer species: factors affecting assessment of transient gene expression using β-glucuronidase reporter gene. *Plant Cell Reports* **12**, 189-193.

Charest, P.J., Devantier, Y. & Lachance, D. (1996). Stable genetic transformation of *Picea mariana* (Black Spruce) via particle bombardment. *In Vitro Cellular and Developmental Biology-Plant* **32**, 91-99.

Cheliak, W.M. & Rogers, D.L. (1990). Integrating biotechnology into tree improvement programs. *Canadian Journal of Forestry Research* **20**, 452-463.

Cheng, Z. M., Schnurr, J.A. & Kapaun, J.A. (1998). Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. *Plant Cell Reports* **17**, 646–649.

Chilton, M.D. (1983). A vector for introducing new genes into plants. *Scientific American* **248**, 50-59.

Christou, P., Ford, T.L. & Kofron, M. (1991). Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important Indica and Japonica varieties via electric discharge particle accelaration of exogenous DNA into mature zygotic embryos. *Bio/Technology* **9**, 957-962.

Christou, P. (1992). Genetic transformation of crop plants using microprojectile bombardment. *The Plant Journal* **2**, 275-281.

Chupeau, M.C., Pautot, V. & Chupeau, Y. (1994). Recovery of transgenic trees after electroporation of Poplar protoplasts. *Transgenic Research* **3**, 13–19.

Comai, L. & Stalker, D. (1986). Mechanisms of action of herbicides and their molecular manipulation. *Oxford Surveys of Plant Molecular Cell Biology* **3**, 166-1955.

Confalonieri, M., Balestrozzi, A. & Bisoffi, S. (1994). Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens. Plant Cell Reports* **13**, 256-261.

Cregg, J.M. & Madden, K.R. (1989). Use of site-specific recombination in regenerate selectable markers. *Molecular and General Genetics* **219**, 320-323.

Crossway, A., Oakes, J.V., Irvine, J.M., Ward, B, Knauf, V.C. & Shewmaker, C.K. (1986). Integration of foreign DNA following microinjection into tobacco mesophyll protoplasts. *Molecular and General Genetics* **303**, 179-185.

Cuenca, B., San-Jose, M.C., Martinez, M.T., Ballester, A. & Vieitez, A.M. (1999). Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. *Plant Cell Reports* **18**, 538–543.

Culafic, L., Budimir, S., Vuficic, R. & Neskovic, M. (1987). Induction of somatic embryogenesis and embryo development in *Rumex acetosella* L. *Plant Cell, Tissue Organ Culture* **12**, 115–125.

Dale, P.J. (1992). Spread of engineered genes to wild relatives. *Plant Physiology* **100**, 13-15.

Dale, P.J., Irwin, J.A. & Scheffler, J.A. (1993). The experimental and commercial release of transgenic crop plants. *Plant Breeding* **111**, 1-22.

Dandekar, A.M., Gupta, P.K., Durzan, D.J. & Knauf, V. (1987). Transformation and foreign gene expression in micropropagated Douglas fir (*Pseudosuga menziesii*). *Bio/Technology* **5**, 587-590.

Dandekar, A.M., McGranahan, G.H., Vail, P.V., Uratsu, S.L., Leslie, C. & Tebets, J.S. (1994). Low levels of expression of wild type *Bacillus thuringiensis* var. *kurstaki cry*IA(c) sequences in transgenic Walnut somatic embryos. *Science* **96**, 151-162.

DeGreve, H., Dhaese, p., Seurinck, J., Lemmers, M., Van Montagu, M. & Schell, J. (1982). Nucleotide sequence and transcript maps of the *Agrobacterium tumefaciens* TI plasmid–encoded octopine synthase gene. *Journal of Molecular and Applied Genetics* **1**, 449-513.

De Jong, A.J., Schmidt, E.D.L. & De Vries, S.C. (1993). Early events in higher-plant embryogenesis. *Plant Molecular Biology* **22,** 367–377.

Dekeyser, R.A., Claes, B., De Rycke, R.M.U., Habets, M.E., Van Montagu, M.C. & Caplan, A.B. (1990). Transient gene expression in intact and organised rice tissues. *Plant Cell Reports* **2**, 591-602.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. & Goodman, H. (1982). Nopaline synthase: Transcript mapping and DNA sequence. *Journal of Molecular and Applied Genetics* **1**, 561-575.

De Paolis, A., Mauro, M.L., Pomponi, M., Cardarelli, M., Sparo, L. & Costantino, P. (1985). Localization of agropine-synthesizing functions in the Tr region of the root inducing plasmid of *Agrobacterium rhizogenes* 1855. *Plasmid* **13**, 1-7.

DeVerno, L.L. & Cheliak, W.M. (1988). *In vivo* transformation of hybrid poplars: clonal variation–a preliminary investigation. In: Molecular Genetics of Forest Trees. Cheliak, W.M. & Yapa, A.C. (eds). Can For. Serv. Petawana Nat. For. Inst. Inf. Rep. PI-X-80. 48-53.

Dhir, S.K., Dhis, S., Savka, m.a., Berlanger, F., Kriz, A.L., Farrand, S.K. & Widholm, J.M. (1992). Regeneration of transgenic Soybean (*Glycine max*) plants from electroporated protoplasts. *Plant Physiology* **99**, 81-88.

Dhir, S.K., Savka, M.A., Berlanger, F., Kriz, A.L., Farrand, S.K. & Widholm, J.M. (1992). Regeneration of transgenic soybean (*Glycine-max*) plants from electroporated protoplasts. *Plant Physiology* **99**, 881–88.

Dodeman, V.L., Ducreux, G. & Kreis, M. (1997). Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany* **48,** 1493-1509.

Douglas, G.C. (1995). Prospects for the advanced propogation and genetic modification of forest species. In: Woods, Trees and Forests in Ireland. Pilcher, J.R. & Mac an tSaoir S.S (eds). 135-57. Royal Irish Academy, Dublin Ireland.

Drake, P.M.W., John, A., Power, J.B. & Davey, M.R. (1997)a. Expression of the *gus A* gene in embryogenic cell lines of Sitka Spruce following *Agrobacterium*–mediated transformation. *Journal of Experimental Botany* **48**, 151–155.

Drake, P.M.W., John, A., Power, J.B. & Davey, M.R. (1997)b. Cytokinin pulse-mediated shoot organogenesiss from cotyledons of Sitka Spruce [*Picea sitchensis* (Bong.) Carr.] and high frequency in vitro rooting of shoots. *Plant Cell, Tissue and Organ Culture* **50,** 147-151.

Draper, J., Davey, M.R., Freeman, J.P., Cocking, E.C. & Cox, B.J. (1982). Ti plasmid homologous sequences present in tissues from *Agrobacterium* plasmid transformed *Petunia* protoplasts. *Plant Cell Physiology* **23**, 451-458.

Draper, A., Scott, R., Armitage, P. & Walden, R. (1988). Plant genetic transformation and gene expression, A laboratory manual, Blackwell Scientific Publications.

Duchesne, L.C. & Charest, P.J. (1991). Transient expression of β-glucuronidase gene in embryogenic callus of *Picea mariana* following microprojection. *Plant Cell Reports* **10**, 191-194.

Duchesne, L.C. & Charest, P.J. (1992). Effect of promotor sequence on transient expression of β-glucuronidase genes in embryogenic calli of *Larix eurolepis* and *Picea mariana* following microprojection. *Canadian Journal of Botany* **70**, 175-180.

Dunstan, D.L., Bekkaoui, F., Pilon, M., Fowke, L.C. & Abrams, S.R. (1988). Effects of abscissic acid and analogues on the maturation of White spruce (*Picea glauca*) somatic embryos. *Plant Science* **58**, 77–84.

DuPleiss, H.J., Oosthuizen, W.T. & Glyn-Woods, C. (1993). Development of plant genetic transformation systems to obtain disease tolerant deciduous fruit trees. *Phytophylactica* **25**, 204-206.

Durzan, D.J. & Gupta, P.K. (1987). Somatic embryogenesis and polyembryogenesis in Douglas fir cell suspension cultures. *Plant Science* 229–235.

Egertsdotter, U. & Von Arnold, S. (1993). Classification of embryogenic cell lines of *Picea–Abies* as regards protoplast isolation and culture. *Journal of Plant Physiology* **141**, 222-229.

Ellis, D., Lazaroff, W.R., Roberts, D.R., Flinn, B.S. & Webb, D.T. (1989). The effects of antibiotics on elongation and callus and bud formation from embryogenic tissue of *Picea glauca*. *Canadian Journal of Forestry Research* **19**, 1340–1342.

Ellis, D.D., McCabe, D.E., McInnis, S., Ramachandran, R., Russell, D.R., Wallace, K.M., Martinell, B.J., Roberts, D.R., Raffa, K.F. & McCown, B.H. (1993). Stable transformation of *Picea glauca* by particle acceleration. *Bio/Technology* **11**, 84-89.

El Maataoui, M. & Espagnac, H. (1987). Neoformation de structures de type embryons somatique sur des cultures de tissue de chene liege (*Quercus suber* fL.). *C.R. Acad. Sc. Paris* **3**, 83–88.

Endemann, M. and Wilhelm, E. (1997). Induction of somatic embryogenesis in *Quercus robur*. Abst Cost 822, Working group 3: Identification and control of phase changes in rejuvenation. Nitra. Slovak Republic., pp 42–44.

Eu, Y.J., Lee, M.H., Chang, H.S., Rhew, T.H., Lee, H.Y. & Lee C.H. (1998). Chlorophyll flourescence assay for kanamycin reistance screening in transgenic plants. *Plant Cell Reports* **17**, 189-194.

Evans, D.A. & Gamborg, O.L. (1982). Chromosome stability of cell suspension cultures of *Nicotiana* species. *Plant Cell Reports* **1**, 104-107.

Everett, N.P., Robinson, K.E. & Mascarenhas, D. (1987). Genetic engineering of Sunflower. *Bio/Technology* **15,** 1201–1204.

Evers, P.W. & Hanish ten cate, Ch.H. (1988). Preliminary exeperiments on transformation of Oak (*Quercus robur*, L.). Abstract poster IUFRO Workshop, Ames, USA.

Evers, P.E., Vermeer, E. & van Eeden, S. (1993). Rejuvenation of *Quercus robur*. *Annals of Scientific Forestry* 50, Suppl. **1**, 330-335.

Feraud-Keller, C. & Espagnac, H. (1989). Condition d'apparition d'une embryogenese somatique sur des calissus de la culture de tissus folaires du chene vert (*Quercus ilex*). *Canadian Journal of Botany* **67**, 1066–1070.

Fillatti, J.J., Sellmer, J., McCown, B., Haissig, B. & Comai, L. (1987). *Agrobacterium*-mediated transformation and regeneration of Populus. *Molecular and General Genetics* **206**, 192-199.

Finnegan, J. & Mc Elroy, D. (1994). Transgene inactivation: Plants fight back! *Bio/Technology* **12**, 883-887.

Flavell, R.B., Dart, E., Fuchs, R.L. & Fraley, R.T. (1992). Selectable marker genes: Safe for plants? *Bio/Technology* **10**, 141-144.

Fowke, L.C., Attree, S.M,. Binarova, P., Galway, M.E. & Wang, H. (1995). Conifer somatic embryogenesis for studies of plant-cell biology. *In Vitro Cellular & Developmental Biology-Plant* **31**, 1-7.

Fraley, R.T., Rogers, S.C., Horsch, R.B., Sanders, P.R., Flick, J.S., Fink, C., Hoffman, N.& Sanders, P. (1983). Expression of bacterial genes in plant cells *Proceedings of the National Acadamy of Science USA* **80**, 4803-4807.

Frame, B.R., Drayton, P.R., Bagnall, S.V., Lewnau, C.J., Bullock, W.P., Wilson, H.M., Dunwell, J.M., Thompson, J.A. & Wang, k. (1994). Production of fertile transgenic Maize plants by silicon carbide whisker-mediated transformation. *The Plant Journal* **6**, 941-948.

Fromm, M.E., Taylor, L.P. & Walbot, V. (1986). Stable transformation of Maize after gene transfer by electroporation. *Nature* **319**, 791-793.

Fujimura, T. & Komamine, A., (1980a). Mode of action of 2,4–D and zeatin on somatic embryogenesis in a carrot cell suspension culture. *Z. Pflanzenphysiol.* **99,** 1 – 8.

Gaskell, G., Martin, W., Bauer, J.D. & Allum, N.C. (1999). Worlds apart? The reception of genetically modified foods in Europe and the U.S. *Science* **285**, 384-387.

Gasser, C.S. & Fraley, R.T. (1989). Genetically engineering plants for crop improvement. *Science* **244**, 1293-1298.

Gammie, J. (1981). World timber in the year 2000. The Economist Intelligence Unit Spec. Rep. **98,** 86 pp.

Genetic Concern Proposal (1999). Ireland A GMO-Free Zone-Niche Markets and a Positive Alternative.

Gerlach, W.L., Llewellyn, D., Haseloff, J. (1987). Construction of a plant disease resistance gene from the satellite RNA of Tobacco ring spot virus. *Nature* **328**, 802-805.

Gilissen, L.J.W., Hanisch-Ten Cate, C.H. & Keen, B. (1983). A rapid method of determining growth characteristics of plant cell populations in batch suspension culture. *Plant Cell Reports* **2**, 232-235.

Gingas, V.M. & Lineberger, R.D. (1989). Asexual embryogenesis and plant regeneration in *Quercus*. *Plant Cell, Tissue and Organ Culture* **17**, 191–203.

Gingas, V.M. (1991). Asexual embryogenesis and plant regeneration from male catkins of *Quercus*. *Hortscience* **26,** 1217–1218.

Godwin, I.D., Ford-Lloyd, B.V. & Newbury, H.J. (1992). *In vitro* approaches to extending the host range of *Agrobacterium* for plant transformation. *Australian Journal of Botany* **40**, 751-763.

Gonzalez, A.E., Schope, C., Taylor, N.J. & Beachy, R.N. (1998). Regeneration of transgenic Cassava plants (*Manihot esculenta* Crantz) through *Agrobacterium*–mediated transformation of embryogenic suspension culture. *Plant Cell Reports* **17**, 827–883

Gressel, J. (1992). Indiscriminate use of selectable markers-sowing wild oats? *TIBTECH* **10**, 382.

Griesbach, R.J. (1985). Advances in microinjection of higher plant cells. *Biotechniques* **3**, 348-350.

Griesbach, R.J. & Hammond, J. (1993). Incorporation of the *GUS* gene into orchids via embryo electrophoresis. *Acta. Horticulturae*. **336**, 165-169.

Gupta, P.K. & Durzan, D.J. (1986). Plantlet production via somatic embryogenesis from subcultured callus of mature embryos of *Picea abies* (Norway spruce). *In Vitro Cellular and Developmental Biology* **22**, 685-688.

Gupta, P.K. & Durzan, D.J. (1987a). Biotechnology of somatic polyembryogenesis and plant regenerationof Loblolly pine. *Bio/Technology* **5,** 147–151

Gupta, P.k. & Duzan, D.J. (1987b). Somatic embryos from protoplasts of Loblolly pine proembryonal cells. *Bio/Technology* **5**, 710–712.

Gupta, P.K., Dandekar, A.M. & Durzan, D.J. (1988). Somatic proembryo formation and transient expression of luciferase gene in Douglas fir and Loblolly pine protoplasts. *Plant Science* **58**, 85-92.

Gupta, P.K. & Pullman, G.S. (1991). Method for producing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. U.S. Patent No. 5,036,007.

Gupta, P.K., Pullman, G., Timmis, R., Kreitinger, M., Carlson, W.C., Grob, J. & Welty, E. (1993). Forestry in the 21st century. *Bio/Technology* **11**, 455-457.

Gupta, P.K., Timmus, R. & Mascarenhas, A.F. (1991). Field performance of micropropagated forestry species. *In Vitro Cellular and Developmental Biology* **27P**, 159-164.

Haggman, H. & Aronen, T. (1998). Transgene expression in regenerating cotyledons and embryogenic cultures of Scots pine. *Journal of Experimental Botany* **49**, 1147-1156.

Hakman, I. & Von Arnold, S. (1985). Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway Spruce). *Journal of Plant Physiology* **121**, 149-158.

Hakman, I., & Fowke, L.C. (1987a). An embryonic cell suspension culture of *Picea glauca* (White spruce). *Plant Cell Reports.* **6,** 20–22.

Hakman, I., Rennie, P. & Fowke, L. (1987). A light and electron microscope study of *Picea glauca* (White spruce) somatic embryos. *Protoplasma* **140**, 100-109.

Halfter, U., Morris, P.C. & Willmitzer, L. (1992). Gene targeting in *Arabidopsis thaliana*. *Molecular General Genetics* **231**, 186-193.

Hammerschlag, F.A. & Smigochi, A.C. (1994). Transgenic Peach plants containing a cytokinin biosynthesis gene displayed altered growth *in vitro* and under greenhouse conditions. *Hortscience* **29**, 454-456.

Hammerschlag, F.A., Zimmerman, R.H., Yadava, U.L., Hunsucker, S. & Geccheva, P. (1997). Effects of antibiotic and exposure to an acidified medium on the elimination of *Agrobacterium tumefaciens* from Apple leaf explants and on the shoot regeneration. *Journal of the American Society for Horticultural Sciences* **122**, 758-763.

Harrison, B.D. (1985). "Advances in gemini virus research". *Annual Review of Phytopathology* **23**, 52-82.

Hartmann, S., Lang, H. & Reuther, G. (1992). Differentiation of somatic embryos from protoplasts isolated from embryogenic suspension cultures of *Abies alba* L. *Plant Cell Reports* **11**, 554-557.

Helmer, G., Casadaban, M., Bevan, M.W., Kayes, L. & Chilton, M.D. (1984). A new chimeric gene as a marker for plant transformation: the expression of *Escherichia coli* β-galactosidase in Sunflower and Tobacco cells. *Bio/Technology* **2**, 520-527.

Hennegan, K.P. & Danna K.J. (1998). pBIN20: An improved binary vector for *Agrobacterium*-mediated transformation. *Plant Molecular Biology* **16**, 129-131.

Herrera-Estrella, L., Depicker, A., Van Montagu, M. & Schell, J. (1983a). Expression of chimeric genes transferred into lants using a Ti plasmid-derived vector. *Nature* **303**, 209-213.

Herrera-Estrella, L., De Block, M.N., Messens, E., Hernalsteens, J.P., Van Montagu, M. & Schell, J.H. (1983b). Chimeric genes as dominant selectable markers in plant cells. *EMBO Journal* **2**, 987-995.

Heutteman, C.A. & Preece, J.E. (1993). Thidiazuron: A potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue, Organ Culture* **33**, 105–119.

Hightower, R., Baden, C., Penzes, E., Lund, P. & Dunsmuir, P. (1991). Expression of antifreeze proteins in transgenic plants. *Plant Molecular Biology* **17**, 1013-1021.

Hille, J., Van Leggan, F., Roelwink, P.J., Frassen, H., Van Kammen, A. & Zabel, P. (1986). Bleomycin resistance; a new dominant selectable marker for plant cell transfromation. *Plant Molecular Biology* **7**, 171-176.

Hobbs, S.L.A., Kpodar, P. & Delong C.M.O. (1990). The effect of T-DNA copy number, position and methylation on reporter gene expression in Tobacco transformants. *Plant Molecular Biology* **15**, 851-864.

Hoekema, A., Hirsch, P.R., Hooykaas, P.J., & Schilperout, R.A. (1983). A binary plant vector strategy based on the separation of the Vir and T region of *Agrobacteria. Nature* **303**, 179-181.

Hoffman, G.A., White, F.F., Gordon, M.P. & Nester, E.W. (1984). Hairy root inducing plasmid; physical map and homology to tumour inducing plasmids. *Journal of Bacteriology* **157**, 269-276.

Holford, P. & Newbury, H. J. (1992). The effect of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhirum majus. Plant Cell Reports* **11**, 93–96

Horsch, R.B., Fry, J.E., Hoffman, N.L., Wallroth, M., Eicholtz, D., Rogers, S.G. & Fraley, R.T. (1985). A simple and general method for transferring genes into plants. *Science* **227**, 1229-1231.

Howe, G.T., Goldfarb, B. & Strauss S.H. (1994). *Agrobacterium*-mediated transformation of hybrid Poplar suspension cultures and regeneration of transformed plants. *Plant Cell, Tissue, Organ Culture* **36**, 59-71.

Huang, Y., Diner, A.M.& Karnosky, D.F. (1991). *Agrobacterium rhizogenes* –mediated transformation and regeneration of *Larix decidua*. *In Vitro Cellular and Developmental Biology Reports* **27**, 201-207.

Humara, J.M., Lopez, M. & Ordas, R.J. (1998). Modifying transient β-glucuronidase expression in pine species using introns. *Plant Cell, Tissue, Organ, Culture* **52**, 183-187.

Hunault, G. & Maatar, A. (1995). Enhancement of somatic embryogenesis frequency by giberellic acid in Fennel. *Plant Cell, Tissue, Organ Culture* **41**, 171–176.

Ide, Y. & Yamamoto, S. (1987). *In vitro* plantlet regeneration from auxillary buds of juvenile seedlings of Konora (*Quercus serrata*). *Journal of the Japanese Forestry Society* **69**, 109-112.

James, D.J., Uratsu, S., Cheng, J., Negri, P., Viss, P. & Dandkar, A.M. (1993). Acetosyringone and osmopectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of Apple . *Plant Cell Reports* **12**, 559-563.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The *GUS* gene fusion system. *Plant Molecular Biology Reporter* **5**, 387-405.

Jefferson, R.A., Kavanagh, R.A. & Bevan, M.W. (1987). Gus fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**, 3901–3907.

Jefferson, R., Goldsbrough, A. & Bevan, M. (1990). Transcriptional regulation of patatin-1 gene in Potato. *Plant Molecular Biology* **14**, 995-1006.

John, A., Drake, P. & Selby, C. (1995). Somatic embryogenesis in Sitka Spruce (*Picea sitchensis* (Bong.) Carr). In: Mohn-Jain, S., Gupta, P.K. & Newton, R.J. (eds.) Somatic embryogenesis in woody plants. Netherlands: Kluwer. 126–143.

Jones, H., Tempelaar, M.J. & Jones, M.G.K. (1987). Recent advances in plant electroporation. *Oxford Surveys of Plant Molecular and Cell Biology* **4**, 347-357.

Jorgenson, J. (1988). Embryogenesis in *Quercus petraea* and *Fagus sylvatica. Journal of Plant Physiology* **132**, 638–640.

Jorgenson, J. (1993). Embryogenesis in *Quercus petraea. Annals of Science and Forestry* **50**, Suppli 1, 344s–350s.

Jorgenson, J. (1993). Embryogenesis in *Quercus petraea. Annual of Science and Forestry* **50**, Suppli 1 344s–350s.

Jorgensen, R. (1993). Silencing of plant genes by homologous transgenes. Proceedings, Royal Society, London.

Joyner, A.L. (1991). Gene targeting and trap screens using embryogenic stem cells: New approaches to mammalian development. *Bioessays* **13**, 649-656.

Kaepplar, H.F., Gu, W., Somers, D.A., Rines, H.W. & Cockburn, A.F. (1990). Silicon carbide fiber-mediated DNA delivery into plant cells. *Plant Cell Reports* **8**, 415-418.

Kermode, A.R., Oishi, M.Y. & Bewley, J.D. (1988). Regulatory roles for desiccation and abscisic acid in seed development: a comparison of the evidence from whole seed and isolated embryos. In: Stanwood P.C., McDonald, M.B. (eds), Seed Moisture. *Crop Science Society Annual.*

Kim, M,S., Schumann, C.M. & Klopfenstein, N.B. (1997). Effects of thidiazuron and benzyladenine on axillary shoot proliferation of three green ash (*Fraxinus pennsylvanica* Marsh.) clones. *Plant Cell, Tissue and Organ Culture* **48**, 45-52.

Kim, Y.W., Lee, B.C., Lee, S.K. & Jang, S.S. (1994). Somatic embryogenesis and plant regeneration in *Quercus acutissima*. *Plant Cell Reports* **13**, 315–318.

Kim, Y.W., Youn, Y., Noh, E.R. & Kim, J.C. (1997). Somatic embryogenesis and plant regeneration from immature embryos of five families of *Quercus acutissima*. *Plant Cell Reports* **16**, 869–873.

Klein, T.M., Wolf, E.D., Wu, R. & Sanford, J.C. (1987). High velocity microprojectiles for delivering nucleic acids into living cells. *Nature* **327**, 70-73.

Klein, T.M., Harper, E.C., Svab, Z., Sanfors, J.C., Fromm, M.E. & Maliga, P. (1988). Stable genetic transformation of intact *Nicotiana* cells by particle bombardment process. *Proceedings of the National Acadamy of Science USA* **85**, 8502-8505.

Klimaszauska, K. (1989). Recovery of somatic embryos and plantlets from protoplast cultures of *Larix X eurolepis*. *Plant Cell Reports* **8**, 440–444.

Kokro, H.I., Karenlampi, S.O. (1998). Transformation of Arctic bramble (*Rubus articus* L.) by *Agrobacterium tumefaciens*. *Plant Cell Reports* **17**, 822-826.

Koop, H.U., Steinmuler, K., Wagner, H., Robler, C., Eibl, C. & Sacher, L. (1996). Integration of foreign sequences into the tobacco plastome via polyethylene glycol-mediated protoplast transformation. *Planta* **199**, 193–201.

Korlach, J. & Zoglauer, K. (1995). Developmental patterns during direct somatic embryogenesis in protoplast culture of European Larch (*Larix decidua* Mill). *Plant Cell Reports* **15**, 242–247.

Koyama, K., Andou, Y., Saruki, K. & Matsuo, H. (1994). Delayed and severe toxicities of herbicide containing glufosinate and a superfactant. *Veterinary and Human Toxicology* **36**, 17-18.

Krogstrup, P., Eriksen, E.N., Moller, J.D. & Rouland, H. (1988). Somatic embryogenesis in Sitka spruce (*Picea sitchensis* (Bong) Carr.). *Plant Cell Reports* **7**, 594–597.

Krogstrup, P. (1990). Effect of culture densities on cell proliferation and regeneration from embryogenic cell suspensions of *Picea sitchensis*. *Plant Science* **72**, 115–123.

Kumpatla, S.P., Teng, W., Buchholz, W.G. & Hall, T.C. (1997). Epigenetic silencing and 5-azacytidine-mediated reactivation of complex transgene in rice. *Plant Physiology* **115**, 361-373.

Lawrence, W.A. & Davies, D.R. (1985). A method for the microinjection and culture of protoplasts at very low densities. *Plant Cell Reports* **4**, 33-35.

Lazzeri, P.A., Brettschneider, R., Lhrs, R. & Lorz, H. (1991). Stable transfomation of barley via PEG- induced direct DNA uptake into protoplasts. *Theoretical and Applied Genetics* **81**, 437–444.

Leple, J.C., Pilate, G., Bottino, M.B. (1993). Transgenic poplars expressing protease inhibitors and a *Bacillus thuringiensis* endotoxin. *International Congress of Plant Pathology* **6**, 189.

Lewin, B. (1990). Genes 4, Oxford University Press.

Li, Y.H., Tremblay, F.M. & Seguin, A. (1994). Transient transformation of pollen and embryogenic tissues of White spruce (*Picea glauca* (Moenck.) Voss) resulting from microprojectile bombardment. *Plant Cell Reports* **13**, 661-665.

Lichtenthaler, H.K. & Buschmann, C. (1984). Das Waldsterben aus botanischer Sicht: Verlauf, Ursachen and Massnahmen. G. Braun, Karlsruhe.

Lloyd, G., & McCown, B. (1980). Commercially–feasible micropropagation of Mountain laurel, *Kalmia latifolia,* by use of shoot–tip culture. *Comb. Proc. Int. Plant Prop. Soc.* **30,** 421–427.

Loopstra, C.A., Stomp, A.M. & Sederoff, R.R. (1990). *Agrobacterium*-mediated DNA transfer in Sugar pine. *Plant Molecular Biology* **15**, 1-9.

Looptrsa, C.A., Weissinger, A.K. & Sederoff, R.R. (1992). Transient gene expression in differentiating pine wood using microprojectile bombardment. *Canadian Journal of Forestry* **22**, 993-996.

Lu, G. & Ferl, R.J. (1992). Site-specific oligdeoxynucleotide binding to Maize Adh 1 gene promoter represes Adh 1-*GUS* gene expression *in vivo*. *Plant Molecular Biology* **19**, 715-723.

Lulsdorf, M.M., Tautorus, T.E., Kikcio, S.I. & Dunstan, D.I. (1992). Growth parameters of embryogenic suspension cultures of Interior Spruce (*Picea glauca-engelmannii* complex) and Black Spruce (*Picea mariana* Mill.)*. *Plant Science* **82**, 227–234.

Machado, Molodo, C., Machado, A. da C., Hanzer, V., Weiss, H., Renger, F., Steinkeller, H., Mattanovich, D., Plail, R., Knapp, E., Kalthoff, B. & Kjatinger, H. (1992). Regeneration of transgenic plants of *Prunus armeniaca* containing the coat protein gene of Plum Pox Virus. *Plant Cell Reports* **11**, 25-29.

Mac An tSaoir, S., O' Brien, J. & Selby, C. (1991). Apparent rejuvenation of mature Sitka Spruce in culture. *Acta. Horticulturae* **289**, 337–338.

Mac Connell, S. (1997). Farmers planted 80% of forestry last year. *The Irish Times*, May 12.

Mac Connell, S. (1999). Only half target being planted, conference told. *The Irish Times*, June 1.

Machida, Y., Usami, A. S., Yamamoto, A., Niwa, Y. & Takabe, I. (1986). Plant inducible recombination between the 25bp border sequences of T-DNA in *Agrobacterium tumefaciens* . *Molecular and General Genetics* **204**, 374-382.

Manzanera, J.A. & Pardos, J.A. (1990). Micropropagation and adult *Quercus suber* L. *Plant Cell, Tissue, Organ Culture* **21**, 1-8.

Manzanera, J.A., Astorga, R., & Bueno, M.A. (1993). Somatic embryo induction and germination in *Quercus suber* L. *Silvae Genetica* **42,** 90-93.

Martinussen, I., Juntilla, O. & Twell, D. (1994). Optimization of transient gene expression in pollen of Norway Spruce (*Picea abies*) by particle acceleration. *Physiologia Plantarum* **92**, 412-416.

Mathews, B.F., Abdul-Baki, A. & Saunders, J.A. (1990). Expression of a foreign gene in electroporated pollen grains of tobacco. *Sexual Plant Reproduction* **3**, 147-151.

Mathias, R.J. & Boyd, L.A. (1986). The effect of cefotaxime on the growth and regeneration of callus from four varieties of Barly (*Hordeum vulgare* L.). *Plant Cell Reports* **6**, 454–457.

Mathias, R, J. & Mucosa, C. (1987). The effect of cefotaxime on the growth and regeneration of callus from four varieties of Barley (*Hordeum vulgare* L.). *Plant Science* **46**, 217-223.

Matzke, M.A., Primig, M., Tronovsky, J. & Matzke, A.J.M. (1989). Reversible methylation and inactivation of marker genes in sequentially transformed Tobacco plants. *EMBO Journal* **8**, 643-649.

Matzke, M.A. & Matzke, A.J.M. (1995). How and why do plants inactivate homologous (trans) genes? *Plant Physiology* **107**, 679-685.

Mc Bride , K.E., Svab, Z., Schaaf, D.J., Hogan, P.S., Stalker, D.H & Maliga, P. (1995). Amplification of a chimeric Bacillus gene in chloroplast leads to an extraordinary level of an insecticidal protein in Tobacco. *Bio/Technology* **13**, 362-365.

Meier-Dinkel, A. (1987). *In vitro* vermehrung und weiterkultur von stieleiche (*Quercus robur*) und traubeneiche (*Quercus petraea*). *Allg. Forst u. Jagdzt.* **158**, 199-204.

Meier-Dinkel, A., Becker, B. & Duckstein, D. (1993). Micropropagation of several clones of late flushing *Quercus robur* L. *Annals of Scientific Forestry* 50 Suppl. **1**, 319-322.

Meyer, P., Linn, F., Heidmann, I., Meyer, H., Niedenhof, I. & Saedlar, H. (1992). Endogenous and environmental factors influence 35S promoter methylation of maize A1 gene construct in transgenic petunia and its colour phenotype. *Molecular and General Genetics* **231**, 345-352.

Michler, C.H. & Bauer, E. (1991). High frequency somatic embryogenesis from leaf tissue of *Populus* spp. *Plant Science* **77**, 111-118.

Mogilner, N., Zutra, D., Gafny, R. & Bar, J.M. (1993). The persistance of *Agrobacterium tumefaciens* in *agroinfected* plants. *Molecular Plant-Microbe Interactions* **6**, 673-675.

Moorhouse, SD., Wilson, G., Hennerty, M.J., Selby, C. & tSaoir, S.M. (1996). A plant bioreactor with medium-perfusion for control of somatic embryogenesis in liquid cell suspensions. *Plant Growth Regulation* **20**, 53-56.

Mowikawa, H. & Yamada, Y. (1985). Capillary microinjection into protoplasts and intranuclear localization of injected materials. *Plant Cell Physiology* **26**, 229-236.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with Tobacco tissue cultures. *Physiological Plant* **52**, 375-379.

Nakano, M. & Mii, M. (1993). Antibiotics stimulate somatic embryogenesis without plant growth regulators in several *Diathus* cultivars. *Plant Physiology* **141**, 721–725.

Nap, J.P., Bijvoet, J. & Stiekema, W.J. (1992). Biosafety of kanamycin-resistant transgenic plants. *Transgenic Research* **1**, 239-249.

Nauerby, B., Katarzyna, B. & Wyndaela, R. (1997). Influence of antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination *of Agrobacterium tumefaciens. Plant Science* **123**, 169-177.

Navarrete, N.E., Van Sambeek, J.W., Preece, J.E. & Gaffney, G.R. (1989). Improved micropropagation of White ash (*Fraxinus americana* L.). In: Rink, G & Budelsky, C.A (eds) Proceedings of the seventh Central Hardwood Conference (pp 146-149). March 5-8. Cabondale, IL.

Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G. & Sala, F. (1987). Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protplasts. *Plant Molecular Biology* **8**, 363-367.

Nelson, R.S., McCormic, S.M., Delanney, X., Dube, P., Layton, J., Anderson, E.J., Kaniewska, M., Proksch, R.K., Horsch, R.B., Rogers, S.G., Fraley, R.Y. & Beachy, R.N. (1988). Virus tolerance, plant growth, and field performance of transgenic Tomato plants expressing coat protein from tobacco mosaic virus. *Bio/Technology* **6**, 403-409.

Ostrolucka, M.G. & Pretova, A., (1991). The occurrence of somatic embryogenesis in the species *Quercus cerris* L. *Biologia* **46**, 9–14.

Ostrolucka, M.G. & Krajmerova, D. (1996). Manifestation of embryogenic potential in culture of zygotic embryos of *Quercus robur* L. *Acta socetatis Botanicorum Poliniae* **65**, 37-41.

O' Sullivan, K. (1998). Forestry policy is flawed, group says. *The Irish Times*, November 23.

Ow, D.W., Wood, K.V., DeLucA, M., DeWet, J.R.., Helinski, D.R. & Howell, S.h. (1986). Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **234**, 856-859.

Park, S.H., Rose, S.C., Zapata, C., Srivatanakul, M & Smith, R.H. (1998). Cross-protection and selectable marker genes in plant transformation. *In Vitro Cellular & Developmental Biology-Plant* **34**, 117-121.

Parsons, T.J., Sinkar, V.P., Stettler, R.F., Nester, E.W. & Gordon, M.P. (1986). Transformation of Poplar by *Agrobacterium tumefaciens*. *Bio/Technology* **4**, 533-536.

Paszkowski, J., Shillto, R.D., Saul, M., Mandak, V., Hohn, T., Hohn, B. & Potrykus, I. (1984). Direct gene transfer to plants. *EMBO Journal* **3**, 2717-2722.

Paszkowski, S. and Saul, M.W. (1986). Direct gene transfer to plants. In: A. Weissbach and H. Weisbach (eds), Methods in Enzymology. Vol. **118**. Academic Press, New York, pp 51 - 62.

Pawlowski, W.P. & Somers, D.A. (1996). Transgene inheritance in plants genetically engineered by microprojectile bombardment. *Molecular Biotechnology* **6**, 17-30.

Peerenboom, E. (1996). Suicidal Potatoes make sacrifice to save crop. *New Scientist* **150**, 20-29

Peterson, R.B. & Aylor, D.E. (1995). Chlorophyll fluorescence induction in leaves of *Phaseolus vulgaris* infected with bean rust (*Uromyces appendiculatus*). *Plant Physiology* **108**, 163-171.

Primach-Zachwiega, S. & Minocha, S.C. (1991). Induction of virulence response in *Agrobacterium tumefaciens* by tissue explants of various plant species. *Plant Cell Reports* **10**, 545-549.

Qiao, J., Kuroda, H., Hayashi, T. & Sakai, F. (1998). Efficient plantlet regeneration from protoplasts isolated from suspension cultures of poplar (*Populus spp.*). *Plant Cell Reports* **17**, 201–205.

Reich, T.J., Iyer, V.N., Scobie, B. & Miki, B.L. (1986a). A detailed procedure for the intranuclear microinjection of plant protoplasts. *Canadian Journal of Botany* **64**, 1255-1258.

Richmond, A.E. & Lang, A. (1957). Effect of kinetin on protein content and survival of detached *Xanthium* leaves. *Science* **125**, 650-651.

Roest, S. & Evers, P.W. (1991). *Agrobacterium*–mediated transformation of oak (*Quercus robur* L.). *Acta Horticulturae* **289**, 259–260.

Sachs, T. & Thimann, K.V. (1964). Release of lateral buds from apical dominance. *Nature* **201**, 939-940.

Santarem, E.R., Trick, H.N., Essig, J.S. & Finer, J.J. (1998). Sonication-assisted *Agrobactreium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. *Plant Cell Reports* **17**, 752-759.

Sarma, K.S., Evans, C. & Selby, C. (1995). Effect of carbenicillin and cefotaxime on somatic embryogenesis of Sitka Spruce (*Picea sitchensis* (Bong) Carr). *Journal of Experimental Botany* **46**, 1779-1781.

Sasaki, Y., Shoyama, Y., Nishioka, I. & Szaki, T. (1988). Clonal propagation of *Quercus acutissima* Caruth by somatic embryogenesis from embryogenic axes. *J. Fac. Agr.*, Kyushu. Univ, **33**, 95–101.

Sasamoto, H. & Hosoi, Y. (1992). Callus proliferation from protoplasts of embryogenic cells of *Quercus serrata*. *Plant Cell, Tissue, Organ Culture* **29**, 241-245.

Sederoff, R., Stomp, A.M., Chilton, W.S. & Moore, L.W. (1986). Gene transfer into Loblolly pine by *Agrobacterium tumefaciens*. *Bio/Technology* **4**, 647-649.

Shackelford, N.J. & Chlan, C.A. (1996). Identification of antibiotics that are effective in eliminating *Agrobacterium tumefaciens*. *Plant Molecular Biology Reports* **14**, 50-57.

Sharp, W.R., Sondahl, M.R., Caldas, L.S., Maraffa, S.B. (1980). The physiology of in vitro asexual embryogenesis. *Horticulture Review* **2**, 268–310.

Sharp, W.R., Evans, D.A., Flick, C.E. & Sommer, H.E. (1983). Strategies and specifications for management of *in vitro* plant propogation. In: Strategies of plant reproduction. Meudt, W.J. (ed). *Beltsville Symp. Agric. Res.* **6**, 287-303.

Shaw, C.H., Leemans, J., Van Montagu, M. & Schell, J. (1983). A general method for the transfer of cloned genes to plants. *Gene* **23**, 315-330.

Sheikholeslam, S.N. & Weeks, D.P. (1987). Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*. *Plant Molecular Biology* **8**, 291-298.

Sheurmann, P. & Dandekar, A.M. (1993). Transformation of temperate woody crops: progress and potentials. *Science Horticulture* **55**, 101-124.

Shimamoto, K., Terada, R., Izawa, T. & Fujimoto, H. (1989). Fertile transgenic rice plants regenerated from transfomed protoplasts. *Nature* **338**, 274–276.

Shin, DI., Podila, G.K., Huang, Y., Karnosky, D.F. (1994). Transgenic Larch expressing genes for herbicide and insect resistance. *Canadian Journal of Forestry Research* **24**, 2059-2067.

Shoyama, Y., Sasaki, Y., Nisioka, I. & Suzaki, T. (1992). Clonal propogation of Oak (*Quercus acutissima* Carruth). In: Y.P.S. Bajaj (eds), Biotechnology in Agriculture and Forestry, Vol, 18, High-Tech and micropropogation II, pp. 179–192. Springer-Verlag, Berlin.

Simola, L.K., & Santanen, A. (1990) Improvement of nutrient medium for growth and embryogenesis of megagametophyte and embryo callus lines of *Picea abies*. *Physiology Plant* **80,** 27–35.

Simpson, R.B., Speilman, A., Margossian, L. & McKnight, T.D. (1986). A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes*. *Plant Molecular Biology* **6**, 403-415.

Skoog, F. & Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symposium for the Society of Experimental Biology* **11,** 118-130.

Smigochi, A., Neal, jr. J.W., McCanna, I. & Douglass, L. (1993). Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the *ipt* gene. *Plant Molecular Biology* **23**, 325-335.

Smith, J.M. & Street, H.E. (1974). The decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota* L.) are serially subcultured. *Annals of Botany* **38**, 223-241.

Smith, D.R. (1991). An automated bioreactor system for mass propogation of *Pinus radiata*. *Agricell Reports* **17**, 1-2.

Songstad, D.D., Somers, D.A. & Griesbach, R.J. (1995). Advances in alternative DNA delivery techniques. *Plant Cell, Tissue and Organ Culture* **40**, 1-15.

Srivastava, P.S. & Steinhauer, A. (1982). In vitro culture of embryo sements of *Quercus lebani:* Organogenesis and callus growth as a differential response to experimental conditions. *Z. Pflanzenphysiol.* **106**, 93-96.

Srivastava, V., Vasil, V. & Vasil, I.K. (1996). Molecular characterisation of the fate of transgenes in transformed Wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* **92**, 1031-1037.

Stachel, S.E., Timmerman, B.. & Zambryskii, P. (1986). Generation of single stranded T-DNA molecules during the initial stages of T-DNA transfer from to *Agrobacterium tumeifaciens* plant cells. *Nature* **322**, 706-712.

Stiekema, W.J. (1992). Raw materials: introducing pest resistance in plants to create "green" raw materials. *Food Biotechnology* **55**, 125-135.

Stomp, A.M., Loopstra, C., Chilton, W.S., Sederoff, R.R., Moore, L.W. (1990). Extended host range of *Agrobacterium tumefaciens* in the genus Pinus. *Plant Physiology* **92**, 1226-1232.

Stomp, A.M., Weissinger, A.K. & Sederoff, R.R. (1991). Transient expression from microprojectile-mediated DNA transfer in *Pinus taeda. Plant Cell Reports* **10**, 187-190.

Syono, K. (1965). Changes in organ forming capacity of carrot root calluses during subculture . *Plant Cell Physiology* **6**, 403-419.

Tautorus, T.E. (1992). Bioreactor culture of Black Spruce and Interior Spruce somatic embryos. Abstract in Proceedings of the third canadian workshop on plant tissue culture and genetic engineering, University of Guelph, Canada. **36**.

Tautorus, T.E., Bekkaoui, F., Pilon, M., Datla, R.S.S., Crosby, W.L., Fowke, L.C. & Dunstan, D.I. (1989). Factors affecting transient gene expression in electroporated Black Spruce (*Picea mariana*) and Jack Pine (*Pinus banksiana*) protoplasts. *Theoretical and Applied Genetics* **78,** 531–536.

Tautorus, T.E., Attree, S.M., Fowke, L.C., & Dunstan, D.I. (1990a). Somatic embryogenesis from immature and mature zygotic embryos, and embryo regeneration from protoplasts in Black Spruce (*Picea mariana* Mill.). *Plant Science* **67,** 115–124.

Tautorus, T.E., Fowke, L.C. and Dunstan, D.I. (1991). Somatic embryogenesis in conifers. *Canadian Journal of Botany* **69**, 1873–1899.

Timberlake, W.E. & Marshall, M.A. (1989). Genetic engineering of filamentous fungi. *Science* **244**, 1313-1317.

Tomes, D.T., Weissinger, A.K., Ross, M., Higgins, R., Drummond, B.J., Schaaf, S., Malone-Schoneberg, J., Staebell, M., Flynn, P., Anderson, J. & Howard, J. (1990). Transgenic tobacco plants and their progeny derived by microprojectile bombardment. *Plant Molecular Biology* **14**, 261-268.

Tremblay, F.M. (1990). Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. *Canadian Journal of Botany* **68,** 236–242.

Trick, H.N. & Finer, J.J. (1997). Sonication-assisted *Agrobacterium*-mediated transformation. *Transgenic Research* **6**, 329-337.

Tsang, E.W.T., David, H., David, A. & Dunstan, D.I. (1989). Toxicity of antibiotics on zygotic embryos of White Spruce (*Picea glauca*) cultured *in vitro*. *Plant Cell Reports* **8,** 214–216.

Tsvetkov, I. (1998). Somatic embryogenesis and regeneration of plantlets in common Oak (*Quercus robur* L.). *Biotechnology and Biotecnological Equipment* **12**, 51-55.

Tzfira, T., Yarnitsky, O., Vainstein, A. & Altman, A. (1996). *Agrobacterium rhizogenes*-mediated DNA transfer in *Pinus halepenis* Mill. *Plant Cell Reports* **16**, 26-31.

USDA Forest Service, (1982). An analysis of the timber situation in the United States, 1952 – 2030. *Forestry Resource Reports* **23**.

Vain, P., Finer, K.R., Egler, D.E., Pratt, R.C. & Finer, J.J. (1996). Intron mediated enhancement of gene expression in Maize (*Zea mays* L.) and Bluegrass (*Poa pratensish*). *Plant Cell Reports* **15**, 489-494.

Van den Elzen, P.J.M., Townsend, J., Lee, K.L. & Bedbrook, J.R. (1985). A chimeric hygromycin resistance gene as a selectable marker in plant cells. *Plant Molecular Biology* **5**, 299-302.

Vieitez, A.M., San-Jose, M.C. & Vieitez, E. (1985). *In vitro* plantlet regeneration from juvenile and mature *Quercus robur* L. *Journal of Horticultural Science* **60**, 99–106.

Vieitez, A.M. & Barciela, S. (1990). Somatic embryogenesis and plant regeneration from embryogenic tissues of *Camellia japonica* L. *Plant Cell, Tissue Organ Culture* **21**, 267–274.

Vilaine, F. & Casse-Delbert, F. (1987). Independent induction of transformed roots by the Tl and Tr regions of the Ri plasmid of agropine type *Agrobacterium rhizogenes*. *Molecular and General Genetics* ***206***, 17-23.

Vitanova, Z., Vitanov, V., Trifanova, A., Savava, D. & Atanasov, A. (1995). Effect of 2,4-D precultivation on regeneration capacity of cultivated Barley. *Plant Cell Reports* **14**, 437–441.

von Arnold, S. (1987). Improved efficiency of somatic embryogenesis in mature embryos of *Picea abies* (L.). Karst. *Journal of Plant Physiology* **128,** 233–244.

von Arnold, S. & Hakman, I. (1986). Effect of sucrose on initiation of embryogenic callus cultures from mature zygotic embryos of *Picea abies* (L.) Karst. (Norway spruce). *Journal of Plant Physiology* **122,** 261–265.

Von Schaevan, A., Stitt, M., Schmidt, R., Sonnewald, U. & Willmitzer, L. (1990). Expression of a yeast derived invertase in the cell wall of Tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic Tobacco plants. *EMBO Journal* **9**, 3033-3044.

Walden, R. (1988). Genetic transformation in plants., Open University Press, Biotechnology series.

Walter, C., Broer, I., Hillemann, D. & Pher, A. (1992). High frequency, heat treatment induced inactivation of the phosphinothricin resistance gene in transgenic single cell suspension cultures of *Medicago sativa*. *Molecular and General Genetics* **235**, 189-196.

Walters, C., Smith, D.R., Connett, M.B., Grace, L. & White, D.W.R. (1994). A biolistic approach for the transfer and expression of a *gus* A reporter gene in embryogenic cultures of *Pinus radiata*. *Plant Cell Reports* **14**, 69-74.

Wan, Y. & Lemeux, P.G. (1994). Generation of large numbers of independently transformed fertile Barley plants. *Plant Physiology* **104**, 37-48.

Wang, Y., Huang, M.R., Wei, Z.M., Jun, Y.R., Chen, D.M., Xu, Z.H., Zhang, L.M. & Xu, N. (1995). Regeneration of Simon Poplar (*Populus simonii*) from protoplast culture. *Plant Cell Reports* **14**, 442–445.

Whetten, R. and Sederoff, R. (1991). Genetic engineering of wood. *Forest Ecology and Management* **43**, 301 306.

Wilson, S., Thorpe, T. & and Maloney, M. (1989). PEG mediated expression of *GUS* and CAT gene in protoplasts from embryogenic suspension cultures of *Picea glauca*. *Plant Cell Reports* 7, 704-707.

Yanofsky, M.F., Porter, S.G., Young, C., Albright, L.M., Gordon, M.P. & Nester, E.W. (1986). The Vir D operon of *A.tumefaciens* encodes a site specific endonuclease. *Cell* **47**, 471-477.

Yibrah, H.S., Manders, G., Clapham, D.H. & Von Arnold, S. (1994). Biological factors affecting transient transformation in embryogenic suspension cultures of *Picea abies*. *Journal of Plant Physiology* **144**, 472-478.

Yoder, J.L. & Gold briugh, A.P. (1994). Transformation systems for generating marker free transgenic plants. *Bio/Technology* **12**, 263-267.

Youvan, D.C., Hearst, J.E. & Marrs, B.L. (1983). Isolation and characterization of enhanced flouescence mutants of *Rhodopseudomonas capsulata*. *Journal of Bacteriology* **154**, 748-755.

Zaghmout, M.F. & Trolinder, N.L. (1993). Simple and efficient method for directly electroporating *Agrobacterium* plasmid DNA into Wheat callus cells. *Nucleic Acid Research* **21**, 1048.

Zaghmout, O.M.F. (1994). Transformation of protoplasts and intact–cells from slowly growing embryogenic callus of Wheat (*Triticum-aestivum* L.). *Theoretical and Applied Genetics* **89**, 577–582.