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Micropropagation of ash (*Fraxinus excelsior L.*).

Thesis presented to the University of Dublin, Trinity College
for the
Degree of Doctor in Philosophy (Ph. D)

by

Jane I. E. Abbott

Department of Botany, Trinity College Dublin
2005

based on research carried out under the joint supervision of

Dr. Trevor R. Hodkinson
Department of Botany, Trinity College Dublin

and

Dr. Gerry C. Douglas
Teagasc, Kinsealy Research Centre, Dublin.

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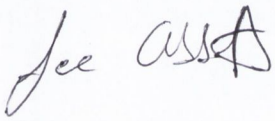

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Table of Contents

Abstract		**1**
CHAPTER 1.	**Introduction**	**4**
1. 1	Justification	4
1. 2.	*Fraxinus excelsior.*	4
1. 3.	Woodland history of Ireland	5
1. 4.	Hardwoods – An agricultural resource	6
1. 4. 1.	The uses and economic importance of ash	7
1. 4. 2.	Improvement of forest tree species	7
1. 5.	*In vitro* culture of plants	9
1. 5. 1.	History	9
1. 5. 2.	Types of tissue culture	9
1. 5. 3.	The advantages and disadvantages of in vitro techniques	10
1. 6.	Micropropagation of hardwood trees	11
1. 6. 1.	Micropropagation of *Fraxinus* species	13
1. 6. 2	Juvenility - maturation problems	15
1. 7.	Plant tissue culture and conservation	18
1. 7. 1.	Short to medium term storage for IVAG	19
1. 7. 2.	Long-term storage or cryopreservation for IVBG	19
1. 8.	Assessing the genetic conformity and diversity of the breeding population	20
1. 8. 1.	Molecular techniques in the assessment of botanical diversity	20
1. 8. 2.	The polymerase chain reaction	20
1. 8. 3.	Random amplified polymorphic DNA	21

1. 8. 4	Variable number of tandem repeats (VNTR) and Simple sequence repeats (SSRs).	22
1. 8. 5.	Restriction fragment length polymorphism	22
1. 8. 6.	Amplified fragment length polymorphism	23
1. 8. 7.	DNA sequencing	23
1. 9.	*In vitro* propagation and somaclonal variation	24
1. 10.	Microsatellite markers for *Fraxinus excelsior*	27
1. 11	Aims/ structure of thesis	27
CHAPTER 2.	**Winter dormancy breaking**	**29**
2. 1.	Introduction	29
2. 2.	Plant dormancy	29
2. 2. 1.	The stages of dormancy	29
2. 2. 2.	Regulation of growth	29
2. 2. 3.	Types of dormancy	33
2. 3.	Breaking bud dormancy	37
2. 4.	Materials and methods	39
2. 5.	Results	42
2. 6.	Discussion	46
CHAPTER 3.	**Establishing shoot cultures *in vitro*.**	**51**
3. 1.	Introduction	51
3. 2.	The initiation of mature *F. excelsior* explants *in vitro*	51
3. 3.	Challenges associated with culture initiation from mature trees	52
3. 4.	Explant selection	53
3. 5.	Sterilisation of explant surfaces	54
3. 6.	Media composition	55

3. 7.	Materials and methods	56
3. 8.	Results	66
3. 9.	Discussion	76
CHAPTER 4.	**Shoot production**	**86**
4. 1.	Introduction	86
4. 2.	Axillary shoot proliferation	86
4. 3.	Culture regime	86
4. 4.	Polyamines as indicators of plant stress *in vitro*	87
4. 5.	Culture media composition	88
4. 5. 1.	Carbohydrate source	90
4. 5. 2.	Plant growth regulators	90
4. 6.	Materials and methods	92
4. 7.	Results	99
4. 8.	Discussion	141
4. 8. 1.	Culture regime	141
4. 8. 2.	Polyamines as indicators of plants stress *in vitro*	142
4. 8. 3.	Culture medium	144
4. 8. 4.	Carbohydrate source	146
4. 8. 5.	Cytokinins	147
4. 8. 6.	Genotypic and physiological factors affecting shoot proliferation	148
CHAPTER 5.	**Root induction and plant growth**	**149**
5. 1.	Introduction	149
5. 2.	Adventitious root development	149
5. 2. 1.	Auxin and root induction	150

5. 2. 2.	The influence of light on rooting *in vitro*	151
5. 2. 3.	The use of cytokinins for root induction *in vitro*	151
5. 2. 4.	Other factors influencing root formation *in vitro*	152
5. 3.	Weaning plants to the *ex vitro* environment	154
5. 4.	Rooting stem cuttings from mature trees	155
5. 5.	Materials and methods	156
5. 6.	Results	163
5. 7.	Discussion	180
5. 7. 1.	IBA exposure time	180
5. 7. 2.	The influence of light on rooting	181
5. 7. 3.	The influence of cytokinin on rooting	182
5. 7. 4.	The influence of media salts on rooting	182
5. 7. 5.	The influence of explant type on rooting	183
5. 7. 6.	The influence of shoot proliferation medium on rooting	183
5. 7. 7.	The effect of harvesting shoots at different times after transfer on rooting	183
5. 7. 8.	Weaning	184
5. 7. 9.	Rooting dormant cuttings	185
CHAPTER 6.	**Analysis of the genetic fidelity of vegetative propagules and the genetic diversity of a sample of the selected population using molecular markers.**	187
6. 1.	Introduction	187
6. 2.	Microsatellite markers and the assessment of diversity	188
6. 2. 1.	Mutation mechanisms of microsatellite loci	189
6. 2. 2.	The mutation rate of microsatellite loci	190
6. 3.	Genetic heterogeneity within organisms	191
6. 3. 1.	The genetic mosaicism hypothesis	192

6. 3. 2.	Plant chimeras and genetic heterogeneity	192
6. 4.	Data analysis	193
6. 4. 1.	Distance and clustering methods	193
6. 4. 2.	Estimation of genetic diversity within the population sample of selected genotypes	194
6. 5.	Material and methods	195
6. 5. 1.	Collection of plant material	195
6. 5. 2.	DNA extraction	198
6. 5. 3	Determination of the fragment size of extracted DNA	199
6. 5. 4.	Spin column purification of the extracted DNA	200
6. 5. 5.	Polymerase chain reaction	200
6. 5. 6.	Visualisation of the PCR products	202
6. 5. 7.	Preparation of samples for analysis on the ABI PRISM™ 310 Genetic Analyser.	202
6. 5. 8.	Data analysis	203
6. 6.	Results	204
6. 6. 1.	Genetic fidelity within genotypes	204
6. 6. 2.	Genetic diversity within the selected population	218
6. 7.	Discussion	222
6. 7. 1.	Genetic fidelity within genotypes	222
6. 7. 2.	Genetic diversity within the selected population	228
CHAPTER 7.	**Summary**	**231**
7. 1.	Introduction	231
7. 2.	Winter dormancy breaking	231
7. 3.	Establishing ash shoot cultures *in vitro*	232
7. 3. 1.	The genotype effect	233

7. 4. Shoot production 233

7. 4. 1. Shoot culture regime and polyamine metabolism 233

7. 4. 2. Bacterial contamination *in vitro* 234

7. 4. 3. Shoot production medium 235

7. 5. Root induction and plant growth 236

7. 6. Genetic fidelity and diversity tests 237

References **239**

Appendices

List of Figures

Fig. 1. 1.	Ash twig showing the black buds.	5
Fig. 1. 2.	Ash foliage.	5
Fig. 2. 1.	Model for G1–S and G2–M transitions in plants.	31
Fig. 2. 2.	Diagram of signals and typical seasons corresponding to the different types of dormancy.	32
Fig. 2. 3.	A flow diagram showing the steps involved in heat treatment of the stem cuttings and forcing in the greenhouse.	41
Fig. 2. 4.	Days to bud burst of dormant winter buds treated for one hour in direct water contact at temperatures ranging from 23°C to 52°C, and forced in the greenhouse, March 2002 and 2003.	45
Fig. 3. 1.	A grafted plant of a selected plus tree growing in the stock plantation at Kinsealy, Co. Dublin.	57
Fig. 3. 2.	An explant with a filter paper pad soaked in 2% Plant Preservative Mixture™ (PPM).	59
Fig. 3. 3.	The three stages involved (post-sterilisation) in establishing a viable shoot culture from dormant winter buds.	60
Fig. 3. 4.	A typical apical and nodal explant taken from naturally flushed shoots of selected 'plus' trees.	62
Fig. 3. 5.	The two types of stem cutting, with the apical bud and without the apical bud, that were heat treated and forced in the greenhouse to produce shoots used for initiating cultures of selected ash trees.	62
Fig. 3. 6.	The effect of heat treatment on viability *in vitro* of dormant apical buds in January, February and March 2002 for selected genotype 7.	69
Fig. 3. 7.	The interaction between genotype (5, 7 and 49), explant type (apical and nodal) and viability (vertical bars denote 95% confidence interval).	72
Fig. 4. 1.	A typical single node explant and shoot tip explant used for *in vitro* shoot production experiments.	93

Fig. 4. 2.	Flow diagram showing the two culture regimes set up to test the effect of culturing on modified MS medium compared to modified OM medium.	98
Fig. 4. 3.	Typical culture jars containing explants of *F. excelsior*.	100
Fig. 4. 4.	Explants of genotype S11 in culture regime 2 suffering from vitrification, spindly leaves and bacterial contamination.	101
Fig. 4. 5.	The concentration of putrescine (nmol g^{-1} FW) in leaf samples from shoots of genotype S11 cultured in three culturing regimes.	102
Fig. 4. 6.	The concentration of spermidine (nmol g^{-1} FW) in leaf samples from shoots of genotype S11 cultured in three culturing regimes.	104
Fig. 4. 7.	The concentration of spermine (nmol g^{-1} FW) in leaf samples from shoots of genotype S11 cultured in three culturing regimes.	105
Fig. 4. 8.	The concentration of putrescine (nmol g^{-1} FW) in leaf samples from shoots of genotype F5 cultured in three culturing regimes.	107
Fig. 4. 9.	The concentration of spermidine (nmol g^{-1} FW) in leaf samples from shoots of genotype F5 cultured in three culturing regimes.	108
Fig. 4. 10.	The concentration of spermine (nmol g^{-1} FW) in leaf samples from shoots of genotype F5 cultured in three culturing regimes.	109
Fig. 4. 11.	Explants of genotype 72 in culture regime 2 suffering from necrosis and spindly leaves.	111
Fig. 4. 12.	The concentration of putrescine (nmol g^{-1} FW) in leaf samples from shoots of genotype 72 cultured in two culturing regimes.	112
Fig. 4. 13.	The concentration of spermidine (nmol g^{-1} FW) in leaf samples from shoots of genotype 72 cultured in two culturing regimes.	113
Fig. 4. 14.	The concentration of spermine (nmol g^{-1} FW) in leaf samples from shoots of genotype 72 cultured in two culturing regimes.	114
Fig. 4. 15	The concentration of putrescine (nmol g^{-1} FW) in leaf samples from shoots of genotype 49 cultured in two culturing regimes.	116
Fig. 4. 16	The concentration of spermidine (nmol g^{-1} FW) in leaf samples from shoots of genotype 49 cultured in two culturing regimes.	117

Fig. 4. 17	The concentration of spermine (nmol g^{-1} FW) in leaf samples from shoots of genotype 49 cultured in two culturing regimes.	118
Fig. 4. 18	The mean putrescine concentration (nmol g^{-1} FW) for genotype S11, F5, 72 and 49 in two culture regimes.	119
Fig. 4. 19	The mean spermidine concentration (nmol g^{-1} FW) for genotype S11, F5, 72 and 49 in two culture regimes.	120
Fig. 4. 20.	The mean spermine concentration (nmol g^{-1} FW) for genotype S11, F5, 72 and 49 in culture regime 2 and 3.	121
Fig. 4. 21.	The concentration of polyamines (nmol g^{-1} FW) in culture regime 1.	122
Fig. 4. 22.	The concentration of polyamines (nmol g^{-1} FW) in culture regime 2.	123
Fig. 4. 23.	The concentration of polyamines (nmol g^{-1} FW) in culture regime 3.	124
Fig. 4. 24.	Shoots of genotype S8 and genotype 47 cultured on modified OM medium.	126
Fig. 4. 25.	Two single node explants of genotype 8 cultured on medium containing 5mg/l BAP and 0.5mg/l TDZ.	134
Fig. 4. 26.	Explants of genotype 49 suffering from excess callus growth and spindly vitrified leaves.	138
Fig. 5. 1.	The bud break scale used for assessing the stage of bud opening in micropropagated plants weaned to the greenhouse.	162
Fig. 5. 2.	The interaction between IBA concentration, genotype and rooting.	164
Fig. 5. 3.	The interaction between genotype, media darkening treatment and rooting.	171
Fig. 5. 4.	The growth of micropropagated plants of three genotypes over two consecutive years after weaning.	178
Fig. 6. 1.	Neighbour joining (NJ) tree of microsatellite data.	206
Fig. 6. 2.	Map of Ireland showing the locations where seed was collected for the provenance trial and the locations of the selected trees used in the genetic diversity study.	219

List of Tables

Table 1. 1.	Summary of developments in tissue culture of *Fraxinus* species.	16
Table 2. 1.	The effect of heat treatment (12 hours at 38°C) given with or without water contact on bud burst in dormant ash buds.	43
Table 2. 2.	The effect of temperature treatments given in water for one hour on bud burst of dormant winter buds in March 2002 and 2003.	44
Table 2. 3.	The effect of heat treatment (37°C for one hour) on bud burst of dormant winter buds of different genotype in March 2003.	46
Table 3. 1.	Sterilisation treatments investigated for dormant winter buds.	64
Table 3. 2.	The effect of sterilisation treatments on contamination levels in cultured dormant apical buds.	68
Table 3. 3.	The effect of pre-sterilisation treatments on contamination in flushed summer shoots.	68
Table 3. 4.	The effect of heat treatment (38°C for 12 hours) on viability and contamination of dormant apical buds of different genotype.	69
Table 3. 5.	The effect of heat treatment (45°C for 1 hour) followed by culture in the light or dark on viability and contamination of dormant apical buds from selected genotype 5 in 2002 and 2003 and wild genotypes in 2003.	70
Table 3. 6.	The effect of culturing heat treated (38°C for 12 hours) dormant apical buds on WPM medium containing five concentrations of glutathione (0 – 5 mg/l).	71
Table 3. 7.	The effect of explant type on viability of flushed summer shoots of different genotype.	72
Table 3. 8.	The effect of initiation time on the viability and contamination rate of flushed summer shoots initiated in June, July and August 2002 (combined results for genotypes 5, 7 and 49).	74
Table 3. 9.	The effect of time of initiation and genotype on the viability of flushed summer shoots *in vitro* in 2002.	74

Table 3. 10.	The effect of time of initiation on viability and contamination rates of flushed summer shoots in 2003 (combined results for genotypes 5, 7 and 49).	74
Table 3. 11.	The effect of initiation time and genotype on contamination rates in flushing summer shoots in 2003.	74
Table 3. 12.	The effect of initiation year on the viability of flushed summer shoots of different genotype initiated in 2002 and 2003.	75
Table 3. 13.	The effect of initiation media on the viability of flushed summer shoots of different genotype initiated in 2002.	75
Table 3.14.	The effect of modified WPM media supplemented with adenine sulphate (15mg/l) and ascorbic acid (10mg/l) compared to unsupplemented media on the viability of flushed summer shoots of different genotype initiated in 2002.	76
Table 3. 15.	Contamination and viability rates for three selected genotypes and one wild genotype initiated from micropropagated mother plants on media with or without 1.1mg/l TDZ.	76
Table 4. 1.	The concentration of four carbon sources tested in M91 medium, to determine the optimum carbohydrate for *in vitro* culture of *F. excelsior.*	97
Table 4. 2.	The concentration and combinations of BAP and TDZ used to test the effect of the two cytokinins on shoot proliferation.	97
Table 4. 3.	The effect of three culture regimes on the propagation rate of shoots of genotype S11.	101
Table 4. 4.	The effect of culture regime on the mean concentration of putrescine, spermidine and spermine in leaf samples of genotypes S11, F5, 72 and 49 over a total of 245 days.	103
Table 4. 5.	The effect of three culture regimes on the propagation rate of shoots of genotype F5.	106
Table 4. 6.	The effect of two culture regimes on the propagation rate of shoots of genotype 72.	110
Table 4. 7.	The effect of two culture regimes on the propagation rate of shoots of genotype 49.	115

Table 4. 8.	Number of new shoots per nodal explant of genotypes S8 and 47 cultured on four types of media (MS, WPM, DKW and OM).	125
Table 4. 9.	Mean shoot length of new shoots produced by nodal explants of genotype S8 and 47 cultured on MS, WPM, DKW or OM.	126
Table 4. 10.	The propagation rate of nodal explants of genotype S8 and 47 cultured on four types of media (MS, WPM, DKW and Ruigini OM).	127
Table 4. 11.	Dry weight increase of genotypes S8 and selected genotype 47 cultured on four types of media (MS, WPM, DKW and OM).	127
Table 4. 12.	Number of new shoots per node, mean shoot length, propagation rate and dry weight increase for explants of genotype 49 cultured on four types of media (MS, WPM, DKW and OM).	128
Table 4. 13.	Viability, number of new shoots per node, mean shoot length, propagation rate and dry weight increase for explants of genotype S8 cultured on M91 media containing four different carbohydrate sources.	130
Table 4. 14.	Viability, number of new shoots per node, mean shoot length, propagation rate and dry weight increase for explants of genotype 49 cultured on media containing four different carbohydrate sources.	130
Table 4. 15.	Viability, number of new shoots per node, mean shoot length, propagation rate and dry weight increase for explants of genotype S11 cultured on media containing four different carbohydrate sources.	131
Table 4. 16.	Viability, number of new shoots per node, mean shoot length, propagation rate and dry weight increase for explants of genotype 49 cultured on media containing four different carbohydrate sources.	132
Table 4. 17.	The viability of single node explants of selected genotypes 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.	133
Table 4. 18.	The number of new shoots produced per node of selected genotype 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.	134

Table 4. 19.	The mean shoot length of the new shoots produced by nodes of selected genotype 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.	135
Table 4. 20.	The propagation rate of nodal explants of selected genotype 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.	136
Table 4. 21.	The dry weight increase of nodal explants of selected genotype 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ	137
Table 4. 22.	The propagation rates for genotype S11 in two culture regimes set up to test the effect of culturing on modified MS medium compared to modified OM medium.	138
Table 4. 23.	The propagation rates for genotype 49 in two culture regimes to test the effect of culturing on modified MS medium compared to modified OM medium.	139
Table 4. 24.	Viability, number of new shoots per node, mean shoot length, propagation rate, dry weight increase and percentage of shoots with callus greater than 5mm in diameter for explants of genotype 54 cultured on two types of modified MS medium and two types of modified OM medium.	140
Table 4. 25.	Viability, number of new shoots per node, mean shoot length, propagation rate, dry weight increase and percentage of shoots with callus greater then 5mm in diameter for explants of genotype 49 cultured on two types of modified OM medium.	141
Table 5. 1.	The number of individual plants of genotype S8, S11, 47 and 49 in each treatment used to test the effect of *a* cold period after successful weaning on bud break and shoot growth.	160
Table 5. 2.	The root induction treatments administered to stem cuttings of dormant micropropagated and grafted plants.	161
Table 5. 3.	Percentage rooting in genotypes S11 and 49 at different concentrations of IBA.	163

Table 5. 4.	Percentage callus > 5mm diameter in genotypes S11 and 49 at different concentrations of IBA.	164
Table 5. 5.	Effect of dark periods (days) on percentage rooting for genotype S11.	165
Table 5. 6.	Effects of pulse exposure to IBA (1mg/l) in darkness for 0 – 8 days on rooting of genotype F5.	166
Table 5. 7.	The effects of pulse treatment with IBA for two days on percentage rooting for genotypes S8 and 47	167
Table 5. 8.	Effect of continuous exposure to IBA on callus production in genotypes S8 and 47.	167
Table 5. 9.	The effects of pulse exposure to IBA for two days in darkness on percentage rooting in genotypes S11 and 47.	168
Table 5. 10. (a)	The number of roots per shoot, total root length and mean root length in genotype S11 after pulse exposure to IBA at different concentrations.	168
Table 5. 10. (b)	The number of roots per shoot, total root length and mean root length in genotype 47 after pulse exposure to IBA at different concentrations.	169
Table 5. 11.	The number of roots per shoot, total root length and mean root length in genotypes 70 and F5 after pulse exposure to 10 mg/l IBA for two days.	169
Table 5. 12.	Percentage rooting in genotypes S8 and 49 after three media darkening methods during continuous exposure to IBA.	170
Table 5. 13. (a)	The number of roots per shoot, total root length and mean root length in genotype S8 three media darkening methods during continuous exposure to IBA.	170
Table 5. 13. (b)	The number of roots per shoot, total root length and mean root length in genotype 49 three media darkening methods during continuous exposure to IBA.	171
Table 5. 14.	Rooting data for shoots of genotype S8 and 49 placed on root induction media with or without 1mg/l BAP.	172
Table 5. 15.	Rooting data for shoots of genotype S8 and 49 (combined) placed on root induction media with or without 1mg/l BAP.	172

Table 5. 16.	Rooting data for shoots of genotype 49 placed on root induction media with or without 1mg/l BAP.	173
Table 5. 17.	The effect of taking shoots for rooting from M91 medium compared to those taken from QRC medium with activated charcoal.	173
Table 5. 18.	The effect of four different nutrient media formulations in the rooting medium, on percentage rooting.	174
Table 5. 19.	The effect of four different nutrient media formulations in the rooting media, on root number.	174
Table 5. 20.	The effect of four different nutrient media formulations in the rooting media, on total root length.	175
Table 5. 21.	The effect on root induction, of taking shoots from genotype S11 at 4, 5, 7 and 9 weeks after transfer to QRC medium with charcoal.	175
Table 5. 22.	The effect on root induction, of taking shoots from genotype 49 at 4, 5, 7 and 9 weeks after transfer to QRC medium with charcoal.	176
Table 5. 23.	The survival rate of rooted and non-rooted shoots of genotype S8, S11, F5, 47 and 49 weaned to the glasshouse.	177
Table 5. 24.	The percentage of plantlets from each genotype in the bud opening categories (0 - 5) from the control and cold period treatments.	177
Table 5. 25.	The effect of rooting treatment, cutting type (apical or nodal) on rooting in cuttings of four genotypes derived from dormant grafted plants.	179
Table 5. 26.	The effect of rooting treatment, cutting type (apical or nodal) on rooting in cuttings of four genotypes derived from dormant micropropagated plants.	180
Table 6. 1.	Source codes used to label DNA samples of selected genotypes of *F. excelsior* collected from different locations and used to study genotypic fidelity during vegetative propagation.	197
Table 6. 2.	The number of plants sampled for nuclear microsatellite DNA analysis of clonal fidelity in *F. excelsior.*	197
Table 6. 3.	Microsatellite primer pairs used in the PCR, including locus name, repeat motif (from original publication), primer sequences and specific annealing temperature (T_a).	201

Table 6. 4.	The cycling parameters (in sequential order) for the amplification of DNA by the microsatellite primers.	201
Table 6. 5.	The fluorescent label colour and name of the microsatellite primers used to analyse the breeding population of *F. excelsior*.	202
Table 6. 6.	The dilution ratio and reaction volume of the fluorescent labelled microsatellite PCR products used for analysis on the genetic analyser.	203
Table 6. 7.	The number samples successfully amplified for nuclear microsatellite DNA analysis of clonal fidelity in *F. excelsior*.	204
Table 6. 8.	The number of alleles and the allelic size range per locus for five microsatellite loci used to test the genetic fidelity within the vegetative propagules of 13 ash trees.	205
Table 6. 9.	The total and mean character differences detected across all loci between samples of genotype F5 (labeled as 05 in this study).	207
Table 6. 10.	The total and mean character differences detected across all loci between samples of genotype 8.	207
Table 6. 11.	The total and mean character differences detected across all loci between samples of genotype 22.	208
Table 6. 12.	The total and mean character differences detected across all loci between samples of genotype 29.	209
Table 6. 13.	The total and mean character differences detected across all loci between samples of genotype 47.	211
Table 6. 14.	The total and mean character differences detected across all loci between samples of genotype 49.	212
Table 6. 15.	The total and mean character differences detected across all loci between samples of genotype 56.	213
Table 6. 16.	The total and mean character differences detected across all loci between samples of genotype 69	213
Table 6. 17.	The total and mean character differences detected across all loci between samples of genotype 70.	215
Table 6. 18.	The total and mean character differences detected across all loci between samples of genotype 72.	216

Table 6. 19. The total and mean character differences detected between samples of genotype 73. 217

Table 6. 20. Allelic diversity of five microsatellite loci scored in a sample of selected genotypes from the Irish population of *Fraxinus excelsior*. 220

Table 6. 21. Allelic diversity of five microsatellite loci scored in a sample *Fraxinus excelsior* trees from an Irish provenance trail. 220

Table 6. 22. Allelic diversity of five microsatellite loci scored in a sample of the natural Irish populations of *Fraxinus excelsior.* 222

Appendix 5

Table 1. The effect of culture regime on the mean putresine content (nmol g^{-1} FW) in leaf samples of genotypes S11, F5, 72 and 49 over 245 days.

Table 2. The effect of culture regime on the mean spermidine content (nmol g^{-1} FW) in genotypes S11, F5, 72 and 49 over 245 days.

Table 3. The effect of culture regime on the mean spermine content (nmol g^{-1} FW) in genotypes S11, F5, 72 and 49 over 245 days.

Appendix 6.

Table 4. The allele lengths (base pairs) of vegetative propagules of genotype F5 for five microsatellite loci.

Table 5. The allele lengths (base pairs) of vegetative propagules of genotype 8 for five microsatellite loci.

Table 6. The allele lengths (base pairs) of vegetative propagules of genotype 18 for five microsatellite loci.

Table 7. The allele lengths (base pairs) of vegetative propagules of genotype 22 for five microsatellite loci.

Table 8. The allele lengths (base pairs) of vegetative propagules of genotype 29 for five microsatellite loci.

Table 9. The allele lengths (base pairs) of vegetative propagules of genotype 44 for five microsatellite loci.

Table 10 The allele lengths (base pairs) of vegetative propagules of genotype 47 for five microsatellite loci.

Table 11. The allele lengths (base pairs) of vegetative propagules of genotype 49 for five microsatellite loci.

Table 12. The allele lengths (base pairs) of vegetative propagules of genotype 56 for five microsatellite loci.

Table 13. The allele lengths (base pairs) of vegetative propagules of genotype 69 for five microsatellite loci.

Table 14. The allele lengths (base pairs) of vegetative propagules of genotype 70 for five microsatellite loci.

Table 15. The allele lengths (base pairs) of vegetative propagules of genotype 72 for five microsatellite loci.

Table 16. The allele lengths (base pairs) of vegetative propagules of genotype 73 for five microsatellite loci.

Abbreviations

ABA	Abscisic acid
AC	Activated charcoal
ADC	Arginine decarboxylase
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
APC	Anaphase-promoting complex
ATP	Adenine tri-phosphate
BA	Benzyladenine
BAP	Benzyladenine purine
PBA	Tetra-hydropyranyl benzyladenine
bp	Base pairs
BR	Brassinosteroids
$Ca(OCL)_2$	Calcium hypochlorite
CAK	CDK-activating kinase
CDKA	Cyclin-dependent kinase
CDKA/B	A- and B-type CDKs
$CuSO_4$	Copper sulphate
CYCA/B	A- and B-type cyclins
CYCD	D-type cyclins
DAF	Amplification Fingerprinting
DFMA	DL-∝-diflouromethylarginine
DFMO	DL-∝-diflouromethylornithine
DKW	Driver and Kuniyuki (1984) walnut medium
DMSO	Dimethyl Sulfoxide
dNTPs	Deoxyribonucleoside triphosphates
DP	Docking protein
DW	Dry weight
F	Fixation index
FAS	Fatty acid synthase
F_{is}	Inbreeding coefficient
GA	Giberellic acid

GABA	γ-aminobutyric acid
GMH	Genetic mosaicism hypothesis
GSH	Reduced glutathione
H_2CN_2	Cyanamide
HC	Hydrogen cyanamide
HCL	Hydrochloric acid
H_E	Expected heterozygosity
$HgCl_2$	Mercuric chloride
H_O	Observed heterozygosity
HSD	Honest significant difference
HSP	Heat shock protein
HWE	Hardy-Weinberg equilibrium
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IBPGR	International Board for Plant Genetic Resources
ICK1	CDK inhibitory protein
IGH	Intraorganismal genetic heterogeneity
ISSR	Inter-microsatellite
IVAG	*In Vitro* Active Gene Banks
IVBG	*In Vitro* Base Gene Banks
JA	Jasmonic acid
KNAP2	KNOTTED-like homeobox protein 2
KNAT2	KNOTTED-like from *Arabidopsis thaliana* 2
MAAP	Multiple Arbitrary Amplicon Profiling
MGBG	Methylglyoxal-*bis*-guanyl hydrazone
mM	Millimoles
MRI	Magnetic resonance imaging
MS	Murashige and Skoog (1962) medium
NAA	Naphthaleneacetic acid
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NJ	Neighbour Joining
NMR	Nuclear magnetic resonance

NS	Non-selected genotypes
ODC	Ornithine decarboxylase
OM	Olive medium (Ruigini, 1984)
PA	Polyamine
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PG	Pholoroglucinol
PGR	Plant growth regulator
PPM	Plant preservative mixture
QL	Quoirin and Lepoivre (1977) medium
QTL	Quantitative trait locus
RAMP	Random amplified microsatellite polymorphism
RAPD	Random Amplified Polymorphic DNA
RB	Retinoblastoma protein
REs	Restriction endonucleases
RFLP	Restriction Fragment Length Polymorphism
Ri	Root inducing plasmid
rol	Root loci
S. E.	Standard error
SAM	S-adenosylmethionine
SCF	SKP1–Cullin–F-box-protein
SNF	Sucrose non-fermenting
SSR	Microsatellite
TDZ	Thidiazuron (1-phenyl-3-(1,2,3-Thidiazol-5-yl)-urea)
TE	Tris EDTA
TIBA	2,3,5-triiodobenzoic acid
UPGMA	Unweighted Pair Group Method using Arithmetic Averages
VNTR	Variable Number of Tandem Repeats
WPM	Woody plant medium (Lloyd and McCown, 1981)

ABSTRACT

Ash (*Fraxinus excelsior* L.) is a native hardwood species in Ireland used as a fast growing tree that is well adapted to agricultural sites. It produces wood valued for its toughness and elasticity. A key objective for its agronomic improvement is to develop means to vegetatively propagate selected mature trees. Seed progeny is heterogeneous, and requires a long period of growing out time before plus trees can be identified. By identifying mature plus trees (selected trees) and vegetatively propagating them it is possible to make testing more efficient and to gain valuable time in tree breeding programs. However, in the mature phase, characterised by flowering and a loss of organogenic competence, most forest species are recalcitrant to standard vegetative propagation methods. *In vitro* culturing methods offer a way around this obstacle and have been assessed in this thesis. Mature ash trees, displaying characteristics desirable for forestry, were phenotypically selected by Collite from sources around Ireland and conserved as grafted plants in Kilmacurra, Co. Wicklow and at Teagasc Kinsealy Research Centre, Co. Dublin. These plants were used in experiments to improve the micropropagation protocols for ash and to develop new methods. The thesis also studied the polyamine production of ash shoots *in vitro* as stress indicators, analysed the genetic fidelity of the vegetative propagules and assessed the genetic diversity of a sample of the selected trees (the breeding population).

In experiments to improve the culture initiation techniques for ash the optimum time of year to initiate cultures from naturally flushed shoots was identified as early in the growing season from May to July. Decontamination of shoots was best achieved by washing thoroughly in running water for two hours before using sterilants, and by a post-sterilisation dip in copper sulphate (100mg/l). The optimum culture medium for the initiation of viable shoots was modified WPM with no plant growth regulators. Nodal explants (40.8%) were more viable *in vitro* than apical explants (2.4%) from naturally flushed shoots. A system was developed using heat treatments (37°C for one hour) applied to dormant stem cuttings followed by forcing under lights to break bud dormancy and produce shoots for culture establishment. The shoots produced by this method were relatively free from contaminants and apical shoots (66.7%) were more viable *in vitro* than axillary shoots (27.1%). The method of shoot forcing was a convenient means to obtain leaf material for DNA analysis.

The polyamine production of ash shoots, during the shoot production phase *in vitro,* was monitored in four genotypes for up to 245 days. Putrescine was identified as a marker for plant stress *in vitro* for four genotypes tested. Furthermore, spermidine and spermine levels were observed to increase in response to abiotic stress in one stress tolerant genotype of ash. Olive medium (OM) was identified as the optimum medium on which to culture ash shoots when compared to modified MS, modified WPM and DKW. Shoots cultured on this medium were healthy and had propagation rates of between 2.2 and

3.3. A high concentration of BAP (5m/l) in combination with TDZ (0.25 - 0.5mg/l) gave a high propagation rate (2.2 – 4.6). Sucrose (30g/l) alone or in an equimolar mixture with mannitol, was identified as the optimum carbohydrate to support the growth of ash shoots *in vitro* compared to glucose or mannitol alone. The optimum culture regime for shoot production was to alternate shoots between modified OM medium containing BAP (5mg/l), TDZ (0.5mg/l), IBA (0.2mg/l) and sucrose (30g/l) and QRC medium containing activated charcoal and sucrose (2g/l). Shoots produced by this system were healthy, free from physiological disorders and had minimal callus production, therefore reducing the risk of introducing somaclonal variation into the culture line.

Auxin concentration and exposure time had a significant effect on rooting in ash. IBA (10-20mg/l) was required for rooting during the induction phase, but inhibited the process during the formation phase. Furthermore, culturing in darkness during the first 2 – 6 days of root induction increased rooting of shoots from a range of genotypes. A pulse treatment of 2-6 days in darkness on IBA followed by transfer to non-IBA containing medium with activated charcoal and culturing in the light was most effective in promoting rooting (up to 85% rooting in mature genotypes). The *in vitro* rooting of ash shoots was found to be dependent not only on the genotype of the explant but also on the physiological state of the shoots at the time of experimentation.

Over 80% of the rooted plantlets were successfully weaned to the glasshouse and they survived outdoors over winter. Furthermore, over wintering outdoors provided the necessary chilling period so that the shoots resumed growth earlier in the season than plantlets kept over winter in a heated glasshouse. It was also demonstrated that 24.7% of ash shoots transferred to *ex vitro* conditions without roots were able to subsequently produce roots and survive weaning to the glasshouse. In experiments on root induction from cuttings of dormant micropropagated weaned plants, rooting was achieved from these plants with or without external auxin application. Maximum rooting was from nodal cuttings with IBA applied to the cut apical surface (75% rooting).

The suitability of microsatellite markers to analyse genetic fidelity of the grafted and micropropagated plants was revealed. Micropropagation techniques produced the least genetic variants within a genotype compared to grafting techniques. Also using microsatellite markers, a relatively high level of genetic diversity was found in a sample of the selected population of ash. The average gene diversity over all loci was 0.88 and the observed number of alleles per locus ranged from 11 to 46. This compared favourably with results from three natural Irish populations but was considerably less than that observed in a sample of provenance trial material with broad geographic range. Therefore, it was concluded that sufficient diversity for a tree improvement program existed between the selected

genotypes but that new material could be incorporated to bring diversity levels in line with an Irish wide sample.

CHAPTER 1. Introduction

1. 1. Justification.

Ash (*Fraxinus excelsior* L.) is a native hardwood species in Ireland but also a fast growing tree well adapted to agricultural sites. It produces wood valued for its toughness and elasticity and is currently the most widely planted commercial broadleaved tree in Ireland. It is the only species suitable for the manufacture of hurleys and also provides wood for the manufacture of furniture and a range of other products (Lally and Fennessey, 2002). A key objective for its agronomic improvement is to develop means to vegetatively propagate selected mature trees that have growth characteristics favourable to commercial forestry. However, most forest species, in the mature phase, are recalcitrant to vegetative propagation. Tissue culture methods offer a way around this obstacle. Mature ash trees displaying superior characteristics such as straight stems, absence of forks, apical dominance and absence of disease were identified and conserved as grafted plants, for use in this project, in the nursery at Teagasc Kinsealy Research Centre (Teagasc, Kinsealy) and also in Kilmacurra, Co. Wicklow. Micropropagation is a feasible option to vegetatively propagate these individual trees. The material can then be deployed to the benefit of Irish farmers in demonstration trials and as stocks for further genetic improvement.

This project therefore aimed to provide a genotype-independent set of conditions and treatments that facilitate the vegetative propagation of selected genotypes of *F. excelsior* by improving known methods and developing new ones for initiating cultures, shoot production, rooting and weaning of selected trees. Clonal lines were tested for variation and a random sample of the selected population compared to the natural Irish population using molecular techniques. The specific aims of each chapter can be found in section 1. 11.

1. 2. *Fraxinus excelsior*

Ash (*Fraxinus excelsior*) belongs to the Oleaceae family and is a native tree found throughout Britain and Ireland. It is a deciduous tree, which reaches an extreme height of 43m; but usually 12-18m, often a shrub in exposed situations (Wardle, 1961). The bark is grey, smooth in young trees and furrowed in mature trees. The buds are black, terminal and 5-10mm long (see figure 1. 1). The leaves are large and compound divided into four to eight pairs of lance-shaped leaflets tipped by a single one, an arrangement that imparts a light feathery arrangement to the foliage (see figure 1. 2) (Wardle, 1961). The flowers are blackish green, small, male, female or hermaphrodite, in dense panicles, appearing long before the leaves (Webb *et al.* 1996). *Fraxinus excelsior* is distributed throughout Europe including Britain and Ireland (see appendix 1), north Africa and western Asia, growing best on loamy soils where both the soil and the atmosphere is moist and cool (Timber Research and Development Association, 1980).

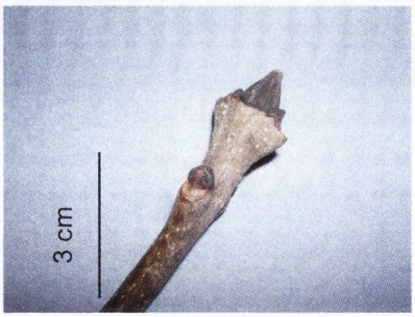


Figure 1. 1. Ash twig showing the black buds.

Figure 1. 2. Ash foliage.

1. 3. Woodland history of Ireland

After the Ice Age, approximately 10,000 years ago, the first response of the flora to warming was a development of rich meadows with grasses, docks (*Rumex*) and meadow sweet (*Filipendula ulmaria*). These were quickly invaded by juniper (*Juniperus communis*) scrub and within 250 years juniper scrub had replaced the meadows over wide regions (Mitchell and Ryan, 1998). About 9,500 years ago, tree willows (*Salix*) began to overshadow the juniper scrub and these in turn gave way to tree birches (*Betula pubescens*); aspen (*Populus tremula*) was also present and Littletonian Ireland had its first woodlands. The birch phase lasted until about 9,250 years ago, by which time pollen of hazel (*Corylus avellana*) appeared in the pollen counts, indicating the arrival of this bush or small tree. The expansion of hazel was rapid in some areas and slower in others; differences in soil and aspect had considerable influence on the establishment of hazel (Mitchell and Ryan, 1998). Pine (*Pinus*) moved into Ireland at about the same time as the hazel and its spread also seems to have been irregular. By 8,500 years ago, the oak and the elm were beginning to overshadow the hazel on the heavier soils and the amount of hazel pollen fell. The pine may have been forced back onto the sandier and drier soils (Mitchell and Ryan, 1998). Alder (*Alnus*) appeared about 7000 years ago. *Fraxinus excelsior* was present but was perhaps confined to dry limestone soils, a habitat which was not sufficiently extensive to enable it to make an effective contribution to the pollen records (Mitchell and Ryan, 1998). Forest returned gradually and by the Atlantic period, approximately 8000 – 5000 BP, forest cover appears to have been well established over the greater part of the Irish land surface, with alder, oak and pine as the main species. With the spread of Bronze Age man 5,000-2,500 BP, primitive agriculture began to reduce the forest area. This process was accelerated by the spread of blanket bog in the cooler and wetter Sub-Atlantic time (McEvoy, 1979).

On the more favourable soils, the disappearance of the broadleaf forest was largely the result of the advance of agriculture, especially the prevention of woodland regeneration by grazing which eliminated seedlings and saplings (McEvoy, 1979). Felling of trees for building and fuel also played

a part. By about 1600AD the fertile plains, especially in the east, were substantially cleared of forest but much still remained in the hill country. Both political and economic considerations led to the subsequent clearance of the hill forests. The political considerations being the need for land for the settlement of colonists and for the elimination of protective cover for the dispossessed Irish (McEvoy, 1979). Economic considerations were the increasing value of timber as an article of commerce, for shipbuilding, house construction and tan bark, as well as for the manufacture of barrels and of charcoal used very extensively in iron smelting (McEvoy, 1979). By about 1700, after the Williamite settlement, the colonists began to feel more secure and planted trees and woods around their estates, using both native and newly introduced species, mostly broadleaf. Oak remained the most profitable tree until after the Napoleonic wars, when tan bark prices fell sharply with the introduction of cheaper substitutes (McEvoy, 1979). From about 1840 onwards, Scots pine (*Pinus sylvestris*) and larch (*Larix*) were widely used and these plantings for coniferous timber production may be regarded as the beginnings of modern Irish forestry. The collapse of the landlord system, which began about 1880, saw the decline of private planting and the felling of many woods. Early in the twentieth century, the state began to assume responsibility for ensuring a home supply of timber. However, only some 1,200 hectares were planted before the Irish State was established in 1922 (McEvoy, 1979).

The Irish Government's 1996 Strategic Plan for the development of the forestry sector is going to literally transform the Irish landscape. It proposes that land under forestry be increased at a rate of 1% every three years over a period of thirty years. The result will be a two and a half fold increase in the area forested in Ireland and with only 9% of land area under forestry (2001), the lowest in the EU, there is much scope to expand our forestry base. Already Ireland has the lowest percentage of broadleaved planting in Europe, where on average 60% of planting is broadleaved. The plan envisages a target of 20% broadleaf plantings, whereas it should be looking towards a 1:1 ratio of conifer planting to broadleaf as recommended by the Heritage Council - the government's independent advisory body on heritage issues (Heritage Council, 1999). Mass planting of non-native conifers on habitats of conservation importance offers little of the benefits of indigenous mixed woodland (Lysaght, 2000).

1. 4. Hardwoods – An agricultural resource

Silviculture includes more and more hardwood species all over Europe. This is partly due to ecological reasons but increasingly also for economical reasons. Timber prices for indigenous hardwoods are steadily increasing, since the tropical hardwood resources for valuable timber are more or less exhausted and the appreciation for domestic hardwoods is rising (Kleinschmit, 1999). Forest trees represent a renewable resource of fibre, chemicals and energy. Forests also hold a firm and necessary place in our conception of what the landscape should be from an aesthetic, watershed, and wildlife habitat perspectives. The steadily increasing use of forest products,

worldwide, forces the view of forests as a crop to be managed and harvested efficiently (Mott, 1986).

1. 4. 1. The uses and economic importance of ash

As a timber tree, *Fraxinus excelsior* is exceedingly valuable not only on account of the quickness of its growth, but for its toughness and elasticity of its wood (Grieve, 1981). Foresters are concerned with ash mainly as a high forest crop for big timber, since if well grown this will fetch high prices (Edlin, 1970). It must, however, be fast grown, because slow grown ash, like slow grown oak, has a high percentage of pore-space and is consequently soft, weak, and brittle. Fast-grown ash is much harder, stronger, heavier and tougher, because its wider annual rings have a greater proportion of structural fibres (Edlin, 1970). There is usually no distinction in colour between sapwood and heartwood, freshly cut wood being whitish to pale brown with a pink caste, turning slightly brownish-white after drying. In some logs an irregular dark brown or black heartwood is found, but this is not necessarily a defect, as the timber is normally quite sound. Logs containing dark heart streaks are often enhanced in value because of the decorative nature of the wood, which is known as olive ash (Timber Research and Development Association, 1980). European ash is typically straight grained, and this, combined with its toughness and flexibility, makes it one of the world's most valuable timbers for such use as sports goods and striking tool handles. Ash also makes excellent logs for burning, giving out no smoke, and the ashes of the wood afford very good potash. Ash bark is astringent and has been employed for tanning nets; both the bark and leaves have medicinal use (Grieve, 1981). It is course textured, and varies in weight according to the growth conditions, from 528kg/m^3 to 816kg/m^3 when dried, but averaging 710kg/m^3 (Timber Research and Development Association, 1980). It is known that ash timber is so elastic that a joist of it will bear more before it breaks than one of any other tree (Grieve, 1981). It matures more rapidly than oak and ash timber always fetches a good price, being next in value to oak. Ash timber is in great demand for quality furniture manufacture, but this demand for quality timber greatly exceeds supply. Europe imported 18,000m^2 of ash wood in the first half of 1988 from the USA (Leg no NO.36, 1988) and the economic value of ash has recently increased considerably in Europe (source: Coillte, 1999). In addition we require 500,000 hurleys per year and some of this production is from imported ash wood.

1. 4. 2. Improvement of forest tree species

While there has been an extensive history for the domestication of food crops, the domestication or improvement of forest tree species has only recently begun (Libby, 1973). The domestication of a wide variety of food crops began around 10, 000 years ago, in many places including the "Fertile Crescent" of South-west Asia, and some evidence suggests that trees were cultivated alongside food crops in traditional agroforestry (Campbell *et al.*, 2003). Tree improvement began in earnest about half a century ago with conventional breeding (Libby, 1973; Zobel and Talbert, 1984). Hardwood forestry can be highly economic, if valuable timber is produced. This production largely

depends on the genetic constitution of the plant material used. The high natural variability of all forest tree species forms an excellent base for selection and improvement (Kleinschmit, 1999).

Trees of value in agriculture and forestry require different strategies in tree improvement. Forest trees continue to require domestication because many of the superior trees have been harvested and the remainder are disappearing from natural stands at an alarming rate (Durzan, 1988). Forest species present some formidable obstacles to rapid genetic improvement (Mott, 1986). These centre primarily on the long period for growth of forest trees and on the pronounced juvenile-mature phases of growth typical of most species. The rapid height growth in the juvenile phase is desired by the forester, since seedlings must develop on competitive shading by the parent canopy, and also of the herbs and shrubs of the forest floor. On the other hand, the prolonged juvenile period before flowering and maturity is a handicap to forest tree breeding programmes. Trees must reach maturity before superior trees can even be identified (Mott, 1986). Elite or superior trees form the basis for tree improvement, they show clean, straight butts, free from knots, as round as possible with the heart straight down the middle, free from wandering heart with minimal taper, without star or ring shake or epicormic burrs. Once identified, the superior trees can be crossed in the hope of producing even more superior individuals among the seed progeny. Accomplishing the desired crosses is not a trivial task, since the trees may be miles apart and cannot be moved, and the flowers usually occur at the treetops far from the unassisted reach of the geneticist (Mott, 1986). After crosses are made and the seeds collected and planted, the progeny trees themselves must go through the long juvenile period to maturity before superior individuals can be selected and before flowers are available for further crosses. The long time to flowering prevents the sorting through many generations quickly and makes inbreeding and backcrossing schemes impossible in reasonable time (Mott, 1986).

A more direct way to capture the special genetic makeup of an elite, mature tree would be through mass vegetative propagation of that particular tree. However, it is characteristic of most forest species that they resist traditional vegetative propagation methods after the mature phase of growth is well underway (Mott, 1986). While a tree is young, cuttings taken from it will root easily. However, as the tree approaches maturity and is old enough to have proven its worth, it also becomes very difficult or impossible to root the cuttings on a commercial scale. Tissue culture methods of propagation offer a way round this obstacle and the promise of larger numbers of propagules in a shorter time than could be obtained with rooted cuttings. Tissue culture methods of micropropagation with apical and axillary buds have been applied successfully to fruit trees in recent years, but application to forest species is less well developed (Mott, 1986). The main advantage of using cell and tissue culture as a tool in breeding programs and mass clonal propagation is its potential for enormous multiplication rates (Thorpe and Biondi, 1984). Thus, while a rooted cutting can produce a single plant from which, several years later, further cuttings are available, even the most limited *in vitro* culture systems – that of resting buds – with today's

techniques can produce several axillary as well as adventitious shoots. Both these types of shoots can in turn be induced to form additional axillary and/or adventitious shoots. The intent of such clonal propagation is to reproduce large quantities of uniform plants of selected qualities (Thorpe *et al.*, 1991).

1. 5. *In vitro* culture of plants

1. 5. 1. History

Shwann (1839) expressed the view that each living cell of a multicellular organism should be capable of independent development if provided with the proper external conditions (Dodds and Roberts 1995; White 1954). A totipotent cell is one that is capable of developing by regeneration into a whole organism and the term was coined by Morgan in 1901 (Krikorian and Berquam, 1969). The first attempts, by Haberlandt in 1902, at plant tissue culture techniques failed. Although earlier workers had achieved *in vitro* culture of orchid seedlings, embryos and plant organs, in 1939 Nobecourt, Gautheret and White (cf. Street, 1973) succeeded in obtaining the first real plant tissue culture. After the Second World War, development in this field was especially rapid and numerous results of importance for agriculture, forestry and horticulture have been published (Pierik, 1979; Bhojwani *et al.*, 1986).

Plant tissue culture lagged behind animal and human tissue culture because of the late discovery of plant hormones (Pierik, 1987a). The first regulator to be discovered, the auxin indole-3-acetic (IAA) acid created great opportunities for the *in vitro* culture of plant tissues. The discovery of the regulator kinetin (a cytokinin) in 1955 was a further stimulus (Pierik, 1987a). Current methods of regenerating plants for rapid propagation are based on manipulating auxin/cytokinin balances to achieve adventitious organogenesis, as disclosed in 1957 by Skoog and Miller; removing high levels of cytokinin, as first accomplished in 1958 by Wickinson and Thimann; and in a very few instances, inducing somatic cell embryogenesis (Murashige and Haung, 1987). The first plant cloning application of tissue culture dates back to 1952, when Morel and Martin attained the elimination of dahlia virus by shoot apex culture. Morel extended the virus exclusion to *Cymbidium* and, in doing so, perceived its ability to rapid clonal propagation of orchids (Morel, 1960). Extension of the tissue culture method to other crops followed the impressive success by commercial orchidologists. The application to other crops began with ferns and ornamentals, then spread slowly to vegetables, fruits, field crops and forest trees (Murashige and Haung, 1987).

1. 5. 2. Types of tissue culture.

De Fossard (1977) differentiated between three types of *in vitro* culture in higher plants:

1. *Organised*: The culture of (almost) whole plants i.e. embryos and seeds, and organ cultures, are termed organised cultures; the characteristic organisational structure of a plant or the individual organ being maintained. It closely resembles *in vivo* vegetative propagation by

cuttings, division, runners and axillary buds or shoots. If the organisational structure is not broken down then progeny arise which are identical to the original plant material (Pierik, 1987a).

2. *Non-organised*: If cells and/ or tissues are isolated from an organised part of a plant, de-differentiate and then are cultured, a non-organised growth in the form of callus tissue results. If the callus disperses clumps of cells (aggregates) and/ or single cells result. Non-organised growth is mainly induced by the use of high concentrations of auxin and/ or cytokinin in the nutrient medium (Pierik, 1987a).
3. *Non-organised/ organised*: This type of culture is intermediate between types one and two. Cells in an isolated organ or tissue, first de-differentiate and then form tissues or a layer of callus tissue, by division, from which organs or even whole individuals often rapidly develop. It must be taken into account that organised structures can develop from non-organised cultures either through special techniques or spontaneously. In all these cases the progeny are often not completely identical with the original plant material (Pierik, 1987a).

1. 5. 3. The advantages and disadvantages of in vitro *techniques.*

According to George (1993) *in vitro* techniques have the following advantages over traditional methods:

- Cultures are started with very small pieces of plants (explants), and thereafter small shoots or embryos are propagated (hence the term 'micropropagation' to describe the *in vitro* methods). Only a small amount of space is required to maintain plants or to greatly increase their number.
- Propagation is ideally carried out in aseptic conditions, free from pathogens. Once cultures have been started there should be no loss through disease, and the plantlets finally produced should be free from bacteria, fungi and other microorganisms (this may not always be the case).
- Methods are available to free plants from virus disease. Providing these techniques are employed, or virus-free material is used for initiating cultures, certified virus-free plants could be produced in large numbers.
- A more flexible adjustment of factors influencing vegetative regeneration is possible such as nutrient and growth regulator levels, light and temperature. The rate of propagation is therefore much greater than in macro-propagation and many more plants can be produced in a given time. This may enable newly selected varieties to be made available quickly and widely, and numerous plants to be produced in a short while. The technique is very suitable when high volume production is essential.
- It may be possible to produce clones of some kinds of plants that are otherwise slow and difficult to propagate vegetatively.

- Plants may acquire a new temporary characteristic through micropropagation, which makes them more desirable to the grower than conventionally raised stock. A bushy habit (in ornamental pot plants) and increased runner formation (strawberries) are two examples.
- Production can be continued all year round and is independent of seasonal changes.
- Vegetatively reproduced material can often be stored over long periods.
- Less energy and space are required for propagation purposes and for maintenance of stock plants (ortets).
- Plant material needs little attention between subcultures and there is no labour or materials requirement for watering, weeding, spraying etc.

Micropropagation is most advantageous when it costs less than traditional methods of multiplication; if this is not the case there must be some other important reason to make it worth while. The chief disadvantages of *in vitro* methods, according to George (1993), are that advanced skills are required for their successful operation; a specialised and expensive production facility is needed; fairly specific methods may be necessary to obtain optimum results from each species and variety; and because present methods are labour intensive, the cost of propagules is usually relatively high. Further consequences of using *in vitro* adaptations are:

- Although they may be produced in large numbers, the plantlets obtained are initially small and sometimes have undesirable characteristics.
- In order to survive *in vitro,* explants and cultures have to be grown in a medium containing sucrose or some other carbon source. The plants derived from cultures are not initially able to produce their own requirement of organic matter by photosynthesis and have to undergo a transitional period before they are capable of independent growth.
- As they are raised within glass or plastic vessels in a high relative humidity, they are not usually photosynthetically self-sufficient; the young plantlets are more susceptible to water loss in an external environment. They may therefore have to be hardened in an atmosphere of slowly decreasing humidity and increased light.
- The chances of producing genetically aberrant plants may be increased.

1. 6. Micropropagation of hardwood trees

Micropropagation is the true to type propagation of a selected genotype using *in vitro* culture techniques (Debergh and Read, 1991). Murashige (1974a, b) proposed a three stage protocol for micropropagation, however as more information has evolved it is now generally accepted that there are five stages critical to successful micropropagation (Debergh and Read, 1991; George, 1993). There is a general consensus that stage 0 (the preparative stage, Debergh and Maene, 1981) is important in the development of a reliable and repeatable micropropagation scheme. Stage 0 involves mother plant selection and preparation. Stock plants selected must be typical of the species or variety and free from any symptoms of disease. Steps to reduce contamination level of

explants are important. Growth, morphogenesis and rates of propagation *in vitro* can be improved by appropriate environmental and chemical pre-treatment of stock plants (George, 1993). Stage 1 involves the initiation of cultures, the goal being to establish an aseptic culture of the selected plant material (George, 1993). The object of stage 2 is to bring about the production of new plant outgrowths or propagules, which, when separated from the culture are capable of giving rise to intact plants (George, 1993). Stage 3 involves preparing the plantlets for growth in the natural environment. Steps are taken to grow individual plantlets capable of carrying out photosynthesis, and survival without an artificial supply of carbohydrate (George, 1993). Stage 3 is often conveniently divided, as Debergh and Maene (1981) suggested, into:

Stage 3a, the elongation of buds or shoots formed during stage 2, to provide shoots of a suitable size for stage 3b;

Stage 3b, the rooting of stage 3a shoots *in vitro* or *extra vitrum*.

Stage 4 involves the transfer of the plantlets to the natural environment. Shoots developed in culture have often been produced in high humidity and low light intensity. Resulting in there being less epicuticular wax than on plants raised in growth chambers or greenhouses. In some plants, the stomata of leaves produced *in vitro* may also be atypical and incapable of complete closure under conditions of low humidity. Tissue culture plants therefore lose water rapidly when moved to external conditions (Sutter and Langhans, 1979, 1980). When supplied with carbohydrates and kept in low light conditions, micropropagated plantlets are not fully dependant on their own photosynthesis. A stimulus which is not provided in the closed *in vitro* environment seems to be needed for them to change to being fully capable of producing their own requirements of carbon and reduced nitrogen (Marin and Gella, 1987).

Pioneering work on the culture of cambial tissues of woody species culminated in the formation of adventitious buds in *Ulmus campestris* in the early 1940s (Gautheret, 1985). Up to 1975, hardwood micropropagation involved the production of callus, and the subsequent regeneration of plantlets from these cultures. Currently organ cultures are being more commonly used (Chalupa, 1987a). Asexual multiplication of both hard and softwoods using tissue culture methods can be achieved by three approaches, namely (1) enhancing axillary bud breaking, (2) the production adventitious buds, and (3) somatic embryogenesis (Thorpe *et al.*, 1991). The first two approaches lead to plantlet formation via organogenesis through the production of unipolar shoots, which must be then rooted in a multistaged process. In contrast, somatic embryogenesis leads to the production of a bipolar embryo, through steps that are often similar to zygotic embryo production (Thorpe *et al.*, 1991).

One of the most striking differences between herbaceous and woody species is that the latter are far more difficult to clone *in vitro*. The reasons for this are (Kunneman-Kooij, 1984; Bonga and Durzan, 1982):

1. Woody species have a relatively weak regenerative capacity when compared to herbaceous species.
2. Research with trees and shrubs was initiated later.
3. The induction of rejuvenation is generally extremely difficult in woody species.
4. The multiplication rate is much lower with woody species than herbaceous species.
5. Dormancy plays a role in the case of trees and shrubs; buds do not open and stem elongation fails to take place.
6. Topophysis (the effect on growth and differentiation of position of axillary buds along the shoot) plays a greater role with woody species.
7. Woody species are more liable to be affected by excretion of toxic substances into the nutrient media.
8. Sterilisation is more difficult with woody species, which are usually grown outside.
9. Trees and shrubs can often only be selected for cloning when they are adult. Since adult material is often very difficult if not impossible to propagate *in vitro*, insurmountable problems are often encountered.
10. Genetic variation in trees is generally greater than in agricultural and horticultural crops, giving rise to variable results.
11. Greenhouse material is not usually available for woody species, and explants must be taken from field grown trees. There will be considerable variation in the explants due to the different growth conditions and annual fluctuations in the climate.

1. 6. 1. Micropropagation of Fraxinus *species.*

There is little practical experience of clonal propagation of forestry *Fraxinus* species, and most ornamental forms have been cloned by grafting onto seedling rootstocks (Hammatt and Ridout, 1992). Chalupa (1990) used nodal segments and shoot tips of ash seedlings as explants and noted that shoot proliferation of cultures was greatly affected by the type and concentration of external cytokinin. Hammatt and Ridout (1992) used embryos extracted from dried *F. excelsior* seeds to study the effects of media and benzyladenine purine (BAP) concentration on micropropagation of ash. They concluded that the basal media used affected culture performance more than BAP concentration and that rooting of shoots was affected by auxin concentration. Hammatt (1994) published the first report of successful micropropagation of a mature ash tree. The main obstacle to establishing cultures was contamination of the initial explants, however once this was overcome it was discovered that satisfactory shoot proliferation was achieved from nodes rather than shoot tip explants. Hammatt (1994) noted the appearance of a *Bacillus* species in the mature ash cultures but this did not affect shoot or root development adversely. It was also noted that with successive subculturing, shoots of the mature clone became easier to root. This occurs in other tree species (Webster and Jones, 1989; Hammatt and Grant, 1993) and may represent rejuvenation during micropropagation. Hammatt (1994) also reports obtaining shoots from the leaflet axils of compound leaves from micropropagated shoots of juvenile and mature ash trees. In this study thidiazuron

(TDZ) was observed to be more effective than BAP in inducing shoot regeneration. Most buds developed from the rachis at the points of attachment of the leaflets, this suggests that compound ash leaves may have evolved by reduction of the stem rather than by progressive lobing of a simple leaf, supporting the partial shoot theory of leaf origin (Arber, 1950). Hammatt (1997) provides a summary of his *in vitro* work with *F. excelsior* seedlings and mature trees. Isolated embryos germinated into seedlings on hormone free media and were micropropagated when transferred to a low salt medium with 22.2 μm BAP. Genotypes in which propagation was not possible from shoot tips were successfully proliferated using nodal explants. In certain genotypes root induction was increased by adding 1mM pholoroglucinol (PG) to the medium containing indole-3-butyric acid (IBA). Micropropagation of two mature trees occurred on media with the same composition as those used for seedlings (Hammatt, 1997). Micropropagated shoots were successfully established in soil; infection of plants with a mycorrhizal fungus at weaning improved growth *ex vitro*. Hammatt (1997) describes the regeneration of shoots from embryo hypocotyls and seedling cotyledons placed on hormone free media containing TDZ. Table 1. 1. (adapted from Hammatt, 1997) (pg. 16) provides a summary of developments in tissue culture of *Fraxinus* species.

Among the other species of *Fraxinus,* plantlets have been obtained from sterilised buds of *F. americana* L. seedlings (Preece *et al.*, 1987) and from embryo derived somatic embryos of *F. americana* (Preece *et al.*, 1989). Bates *et al.* (1992) reported that the artificial cytokinin TDZ stimulated shoot organogenesis and somatic embryogenesis in *F. americana*. The effects of TDZ and BAP on axillary shoot proliferation of green ash (*F. pennsylvanica* Marsh.) was investigated by Kim *et al.*, (1997) they report that the concentration of both TDZ and BAP affect shoot biomass and significant clonal differences were noted in the proliferation of axillary shoots. To ensure clonal fidelity low levels of cytokinin are recommended by this study for stable shoot proliferation as high levels may result in the formation of both axillary and adventitious shoots. Micropropagation of juvenile and adult flowering ash (*Fraxinus ornus* L.) has been developed using shoot apices or nodal segments from aseptically grown seedlings and shoot apices from adult trees (Arrillaga *et al.*, 1992). In this study it was noted that shoots produced by the recultured explants from the mature trees exhibited responses similar to that of juvenile material. Micropropagation of juvenile and mature plant material from *F. angustifolia* Vahl. was achieved using shoot tip and nodal explants (Perez-Parron *et al.*, 1994). The mature material was collected from dormant trees during winter. Cuttings were placed in water with or without growth regulators and it was discovered that percentage bud sprouting was higher with water on its own (24%) or with 4.4μm benzyladenine (BA) (28%) than with 5.4μm naphthaleneacetic acid (NAA) (18%) or 14.4μm Gibberellic acid (GA_3) (8%). Buds from the elongated sprouts were used as explants. For both juvenile and mature material the proliferation rate was highest on a low salt medium, this was also reported by Arrillaga *et al.* (1992) in *F. ornus*.

1. 6. 2. Juvenility- maturation problems.

Increased success in rooting with successive subculturing occurs in other tree species apart from ash (Sriskandarajah *et al.*, 1982; Webster and Jones, 1989; Hammatt and Grant, 1993). Enhanced competence to root and greater vigour in some micropropagated mature trees (Jones and Hadlow, 1989) may represent rejuvenation during micropropagation. True rejuvenation includes the complete reversal of maturation as a result of sexual reproduction or vegetative propagation via adventitious shoot formation or somatic embryogenesis. There is uncertainty regarding the possibility of obtaining true rejuvenation, therefore the term should perhaps be replaced with the term reinvigoration (Pierik, 1990). Explanations given for rejuvenation are often vague and speculative, since little is known about the physiological and biochemical factors causing rejuvenation. Isolated meristems may rejuvenate as a result of wounding or as a result of loss of correlative control, the reduction in the number of cells when a plant is formed via meristem culture is thought to be the cause of rejuvenation (Pierik, 1990).

Phase specific biochemical markers could provide a means of monitoring progress during a rejuvenation protocol and possibly discriminate between reinvigoration and rejuvenation (Von Aderkas and Bonga, 1998). Various proteins have been identified that are unique to juvenile shoots (*Sequoiadendron*; Bon, 1988, Bon and Monteuuis, 1991) or show higher activities in juvenile shoots than in adult shoots (*Sequoia sempervirens*; Berthon *et al.*, 1987). Compared with adult hybrid walnut trees, terminal shoots of rejuvenated hybrid trees were associated with increased phenylalanine ammonia-lyase (PAL) and chalcone synthase activities and a high hydrojuglone glucoside to myricitrin ratio (Claudot *et al.*, 1993). In hazel nut (*Corylus* spp.) trees, partially rejuvenated by severe pruning, free putrescine concentration increased, whereas concentrations of free spermidine and spermine decreased (Rey *et al.*, 1994).

As a tree passes from the juvenile to the adolescent and then to the mature phase, the ability to modify tissues is largely lost and the rooting of cuttings and tissue culture become very difficult. One of the major problems preventing a wider use of micropropagation is the inability to manipulate easily *in vitro* explants taken from mature trees (Thorpe *et al.,* 1991). Most of the success reported involves the use of excised embryos and seedling parts as explants. The reason for this choice is that by the time trees are old enough for evaluation, they are often recalcitrant in culture. Thus, propagation is carried out with unproven material, even if full-sib seed, arising from controlled pollination is used. Since most tree species are outbreeders, much genetic variation usually exists in any seed population (Thorpe *et al.,* 1991).

Table 1. 1. Summary of developments in tissue culture of *Fraxinus* species (adapted from Hammatt, 1997).

Species	Studies	Reference
F. excelsior	Micropropagation of juvenile shoots.	(Chalupa, 1983; 1990; Leforestier *et al.*, 1990; 1991; Hammatt and Ridout, 1992; Silveira and Cottignies, 1994)
	Micropropagation of mature shoots	(Hammatt, 1997; 1994) (Pierik and Sprenkels, 1997)
	Adventitious shoot formation from:	
	1. hypocotyls	(Tabrett and Hammatt, 1992)
	2. cotyledons	(Hammatt, 1997)
	3. leaves	(Hammatt, 1994)
	4. petioles	(Hammatt, 1997)
F. americana	Micropropagation of juvenile shoots	(Heiman and Preece, 1983)
		(Preece *et al.*, 1987)
		(Navarrete *et al.*, 1989)
	Establishment of mature buds *in vitro*	(Browne and Hicks, 1983)
	Somatic embryogenesis from seed explants	(Preece *et al.*, 1987; Preece *et al.*, 1989)
	Adventitious shoot formation from seed explants	(Bates *et al.*, 1992)
F. angustifolia	Micropropagation of juvenile and mature shoots	(Perez-Parron *et al.*, 1994)
	Shoot organogenesis from seed embryos	(Tonon *et al.*, 2001a)
	Somatic embryogenesis from immature zygotic embryos	(Tonon *et al.*, 2001b)
F. ornus	Micropropagation of shoots	(Leforestier *et al.*, 1991)
	Micropropagation of juvenile and mature shoots	(Arrillaga *et al.*, 1992)
F. pennsylvanica	Establishment of juvenile buds *in vitro*	(Heiman and Preece, 1983; Preece *et al.*, 1987)
	Micropropagation of	(Einset and Alexander, 1985)
	seedlings	(Kim *et al.*, 1997)
	Callus formation from internode explants	(Preece *et al.*, 1987)

All woody plants exhibit phase changes. These include morphological alterations, as exemplified by phyllotaxy or leaf form (Gupta *et al.*, 1991), and physiological phase changes, e.g. differences in anthocyanin production (Murray *et al.*, 1994). Other responses that show marked differences between juvenile and adult stages include inducibility of organogenesis and embryogenesis in tissue culture, with juvenile tissues such as embryonic stages and young seedlings being the most responsive (Greenwood, 1995). Loss of organogenesis and embryogenesis (competence), whether progressive or sudden, reflects transition to the mature phase (Von Aderkas and Bonga, 1998). The goal is to break out from these strictures and find ways of multiplying plants from mature tree tissues. Since it is easier to propagate juvenile than mature material, two options are available, namely (a) select the most juvenile tissues within a tree, and (b) rejuvenate parts of the donor tree by special treatments prior to excision (Bonga, 1987). Cutting from lower branches close to the trunk, orthotropic shoots that originate from the base of the trunk and shoots that develop from sphaeroblasts are more juvenile than branches from other parts. Thus, the use of stump sprouts and the practice of coppicing are common (Thorpe *et al.,* 1991). Reactivation can also be achieved by serial grafting, e.g. repeated grafting of scions from mature trees onto seedling root stocks accentuated and prolonged juvenile behaviour in the scions in *Hevea (*rubber), *Eucalyptus* and *Pseudotsuga* (Douglas Fir) (Franclet *et al.*, 1987). Etiolation of branches prior to excising shoot tips has also been useful. Similarly, serial rooting of mature cuttings has produced scions with more juvenile traits in both soft and hard woods (Thorpe *et al.,* 1991).

In vitro treatment of plant material can sometimes bypass the phase change limitations on propagation (Aderkas and Bonga, 1998). A process called micrografting, in which meristems are removed from older trees and grafted on aseptically raised seedlings, has been used to multiply *Thuja plicata* J. Donn *ex* D. Don (Mission and Giot-Wirgot, 1984) and *Larix decidua* Mill. (Ewald and Kretschmar, 1996). Micropropagation of some species is enhanced by the use of *in vitro* derived material. Ruaud *et al.* (1992) showed that seedlings and young plants derived from somatic embryos were more juvenile than their similarly aged zygotic counterparts. Furthermore, somatic embryogenesis was more readily induced from mature plants derived by somatic embryogenesis than from plants of seedling origin (Paques *et al.*, 1997).

Maintenance of juvenility depends on *in vitro* conditions. Kretzschmar and Ewald (1994) serially cultured shoot tips of 140 year-old larch. After 10 subcultures it was possible to achieve adventitious root regeneration, but better rooting and plant survival occurred after 14 subcultures when the needles exhibited juvenile morphology. Cuttings from 3 year-old micropropagated plants of this larch had a 10 fold-improvement in rooting over that of cuttings from the original ortet. Oak (*Quercus*) is more difficult to rejuvenate *in vitro* than larch. Ewald and Naujoks (1997) reported that, in oak, explant origin was more important in controlling subsequent behaviour than any tissue culture treatment. Many other studies have shown that, in addition to the use of appropriate *in vitro*

conditions, the choice of explant is important when attempting organogenesis or embryogenesis in tissues from mature trees.

Another major problem encountered mainly with temperate hardwoods is the episodic growth pattern observed in culture (McCown and McCown, 1987). When explants or shoots from mature specimens are placed in culture, three distinct phases can be identified. These are an isolation phase, followed by a stabilisation and eventually a production phase. During the isolation phase, growth is rapid and the few axillary shoots that are produced are usually an expression of buds already present on the original explant. These cultures then become quiescent for a period of time depending on the maturity of the starting material. During the stabilisation phase, growth is dependent on meristems generated in culture, and juvenile meristems seem to respond best. During the production phase, growth and the quality of shoots do not change with subculturing, and shoots can be rooted and acclimatised (Thorpe *et al.,* 1991). McCown and McCown (1987) classified hardwood trees into three groups based on the ease of stabilisation in culture. For example, *Populus, Salix* and *Ulmus* respond readily, and are stabilised in a short period; *Acer* and *Fraxinus* are stabilised with some difficulty and *Quercus* and *Fagus* species are the most difficult to stabilise and require a long period to do so.

1. 7. Plant tissue culture and conservation.

There are two basic approaches to the conservation of plant genetic resources, *ex situ* and *in situ*, and they are complementary. There are also various methods for the conservation of genetic resources. The best way is to adopt a combination of cost effective and practical methods, to conserve the targeted gene pool of a species (Ng and Ng, 2002). Maintenance and preservation of plant germplasm in field genebanks is difficult, expensive and labour intensive. Conservation of seeds has been practised for a long time but it suffers from severe limitations like low seed viability and heterozygosity. Therefore a need was felt to develop alternative methods, viz., *in vitro* preservation (Kartha, 1981). New techniques help to conserve germplasm disease-free, but also involve lower labour costs and requirement for technical personnel besides limiting disease-transfer (Withers and Engelmann, 1990).

An IBPGR (International Board for Plant Genetic Resources) committee laid the theoretical groundwork for *in vitro* genebanks in the early 1980's. It found that *in vitro* technology offered an efficient means of storing both vegetatively propagated crops -such as banana (*Musa* X *paradisiaca* L.), cassava (*Manihot esculenta* Crantz.) and sweet potato (*Ipomoea batatas* L.) - and plants that produce recalcitrant seeds (seeds that are not easily stored by conventional methods such as drying and freezing) (Thomas, 1986).

According to Sahijram and Rajasekharan (1997) the properties required of a successful storage system are the ability to:

- Reduce the growth rate of the plants, resulting in extension of intervening sub-culture frequencies.
- Retain plant viability during storage at maximum simultaneously maintaining genetic stability at minimum.
- Maintain the full developmental and functional potential of stored material when it is returned to physiological temperature.
- Make significant savings in labour input, materials and commitment to specialised growing facilities.

When deciding upon a conservation strategy employing tissue culture systems, the relative advantages of each system must be evaluated (Ford-Lloyd and Jackson, 1986). A system which provides optimum storage conditions may not be entirely appropriate for plant regeneration after storage, and *visa versa*. The genetic stability and potential for both quantitative losses and qualitative changes are all factors which determine which tissues to utilise and the most appropriate storage conditions. Undoubtedly freeze preservation offers the best hope for long- term storage (Ford-Lloyd and Jackson, 1986).

In vitro methods are different from either the *in situ* conservation in natural habitat or *ex situ* conservation in field conditions. Tissue cultures may be conserved at either relatively low temperature of 15 to 20°C (slow growth) or cryopreserved in liquid nitrogen at –196°C (no growth). Accordingly, *In Vitro* Active Gene Banks (IVAG) and *In Vitro* Base Gene Banks (IVBG) may be established, respectively (Sahijram and Rajasekharan, 1997).

1. 7. 1. Short to medium term storage for IVAG

The basic principle underlying this form of storage is the induction of reduced vegetative growth of the stored material by inducing osmotic stress, limiting the availability of carbohydrate to sub-optimal levels, low temperature/dark maintenance of cultures or incorporating growth retardants in the culture medium. These methods may be used singly or in combination (Sahijram and Rajasekharan, 1997).

1. 7. 2. Long-term storage or cryopreservation for IVBG

In this method, the cultures are suspended at very low temperatures (–196°C) in liquid nitrogen to facilitate the arrest of mitotic and metabolic activities. The procedure guarantees long-term preservation of germplasm in genetically unaltered state. The chief drawback, however, is that the viability of very few biological materials can remain unaffected when they are frozen at very low temperatures. Cryoprotectants such as Dimethyl Sulfoxide (DMSO) can help and are regularly used (Sahijram and Rajasekharan, 1997). Brearley *et al.* (1995) demonstrated that cryopreservation was possible with zygotic embryos of *Fraxinus excelsior*.

In the case of apple (*Malus* spp.) and banana (*Musa* spp.), *in vitro* conservation using synthetic seed (synseed) has been demonstrated. This procedure involves the formation of sodium alginate beads around the explant to afford desiccation tolerance for prolonged periods. In a previous definition, the only explant type that was considered for synseed production was the somatic embryo, which is a bipolar structure (Redenbaugh *et al.*, 1993). Somatic embryogenesis, though, has not been achieved in all species, and micropropagated buds (unipolar explants) offer an interesting alternative (Micheli *et al.*, 1998). Encapsulation of *in vitro* proliferated buds of olive (*Olea europaea* L.) has been achieved (Micheli *et al.*, 1998) and it is also used in Kiwifruits as a means of conserving germplasm (Adriani *et al.*, 2000). The application of *in vitro* methods to plant genetic conservation has additional advantages. During the culturing of vegetatively propagated crops, many serious diseases, particularly viruses can be eliminated and disease free stocks maintained (Henshaw, 1984). This can have important implications for germplasm exchange and international plant quarantine. Tissue culture also has a major advantage in that a large number of genotypes can be stored in a relatively small area and generally at a fraction of the cost of growing material annually in the field, or maintaining large living collections of trees (Ford-Lloyd and Jackson, 1986).

1. 8. Assessing the genetic conformity and diversity of the breeding population.

Chapter six of this thesis uses molecular methods to assess genetic variation in a selected population of *F. excelsior* and to study the genetic stability during micropropagation. The following section discusses the main marker systems used in diversity evaluation.

1. 8. 1. Molecular techniques in the assessment of botanical diversity

Traditional approaches to the measurement of botanical diversity rely upon the ability to resolve differences in morphological characters; the range of characters available has increased by the use of electron microscopy, biochemical and photochemical assays. Although the new wave of data emerging from the DNA-based techniques reveals an even greater range of characters, they are not necessarily superior to those revealed by the more traditional methods. Molecular techniques vary in the way that they resolve genetic differences, in the type of data they generate and in the taxonomic levels at which they can be most appropriately applied (Karp *et al.*, 1996).

1. 8. 2. The polymerase chain reaction (PCR)

The development of the polymerase chain reaction (PCR) led to a revolution in the applicability of molecular methods and a range of new technologies were developed employing the PCR based marker systems (Burke, 1996). The polymerase chain reaction is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA. In the reaction there are three nucleic acid segments: the segment of double-stranded DNA to be amplified and two single stranded oligonucleotide primers flanking it. Additionally there is a protein component (a DNA polymerase), appropriate deoxyribonucleoside triphosphates (dNTPs), a buffer, and salts. The primers are added in vast excess compared to the DNA to be amplified. They hybridise to opposite strands of the

template DNA and are orientated with their 3' ends facing each other so that synthesis by DNA polymerase (which catalyses growth of new strands 5'$\longrightarrow$3') extends across the segment of DNA between them. One round of synthesis results in new strands of indeterminate length which, like the parental strands, can hybridise to the primers upon denaturation and annealing. These products accumulate only arithmetically with each subsequent cycle of denaturation, annealing to primers, and synthesis. The second cycle of denaturation, annealing, and synthesis produces two single-stranded products that together compose a discrete double-stranded product which is exactly the length between the primer ends. Each strand of this discrete product is complementary to one of the two primers and can therefore participate as a template in subsequent cycles (Coen, 1997).

1. 8. 3. Random Amplified Polymorphic DNA (RAPD)

RAPDs have been commonly used for estimating genetic relationships among closely related populations or species of plants (Rieseberg, 1996). RAPD analysis involves fragments of DNA being synthesised in a PCR reaction mixture containing genomic DNA and an arbitrarily chosen primer that has binding sites on the complementary strands of the genomic DNA within approximately 3kb (Yu and Pauls, 1994). The primer used in RAPD analysis is short (usually 10 base pairs (bp) long) with a GC content of between 50 and 80% and a relatively low annealing temperature of 34 – 37°C is used during the PCR reaction (Penner, 1996). Using a short primer increases the probability that the primer will anneal at priming sites close to one another and on opposite DNA strands. The reaction conditions allow scanning of the entire genome for these small primer sites. The amplification product is the intervening region of DNA, of variable length, these are traditionally separated by electrophoresis on an agarose gel stained with ethidium bromide (Bradley, 1999). The data derived from RAPDs have their strength in distinguishing individuals, cultivars or accessions. The presence or absence of bands can be scored and the data converted into similarity matrices for calculation of genetic distance. According to Karp *et al.* (1996) the enormous attraction of this arbitrary priming technique is: (a) no requirement for DNA probes or sequence information for the design of specific primers; (b) the procedure is quick, simple and automatable and; (c) very small amounts of DNA (10ng per reaction) are required. RAPDs also have their limitations and when using arbitrary priming techniques it must be realised that: (a) the markers are co-dominant and heterozygotes cannot be detected; (b) in the absence of pedigree analyses, the identity of individual bands in the multi-band profiles is not known and there can be uncertainty in assigning markers to specific loci; (c) the presence of a band of apparently identical molecular weight in different individuals cannot be taken as evidence that the two individuals share the same homologous fragment, although this assumption is commonly made and; (d) single bands on the gel can sometimes be comprised of several co-migrating amplification products (Karp *et al.*, 1996). Also RAPD analysis is difficult to reproduce within and between laboratories, it is important to maintain strictly constant PCR reaction conditions in order to achieve reproducible profiles.

1. 8. 4 Variable number of tandem repeats (VNTR) and simple sequence repeats (SSRs).

Mammalian and plant genomes contain large amounts of repetitive DNA sequences, an increasing number of which are being identified as stretches of tandem repeat units. The repeat units ranging in size from 8 to 50bp form stretches of DNA referred to as variable number tandem repeats or minisatellites. The repeated units ranging in size from 2 to 6 bp form stretches of DNA referred to as short tandem repeats or microsatellites (SSR). Microsatellites have been particularly useful for comparative genetics and genome mapping as they show a high degree of length polymorphism (Oh and Mao, 1999). A different allele occurs at an SSR locus as a result of changes in the number of times a core element is repeated, altering the length of the repeat region. Differences in length at an SSR locus are detected with DNA amplification by the PCR using two oligonucleotide primers that complement unique sequences flanking the SSR locus. SSR markers are co-dominant, highly abundant and only require a small quantity of template DNA for amplification, they also give highly reproducible results (Jauhar, 1996). Disadvantages of SSR include uncertainty about the functional role of nucleotide repeat variation, the work required to develop primers for each new species examined and the fact that only a few allelic states are possible enhancing the chance of parallel evolution of a particular length variant, this particular problem means that phylogenetic analysis of the alleles discovered is difficult or misleading (Palumbi, 1996). However, they have a proven record in the genotyping of cultivated plants and have been shown to be more sensitive than RAPDs as a means of characterising complex genomes (Chapman *et al.*, 2000).

A variant of the SSR technique described by Zietkiewicz *et al.* (1994), uses as primers microsatellite oligonucleotides that amplify genomic segments different from the repeat region itself. This approach, named Inter-SSR (ISSR), employs oligonucleotides based on a simple sequence repeat anchored or not at their 5'- or 3'-end by two to four arbitrarily chosen nucleotides. This triggers site specific annealing and initiates PCR amplification of genomic segments which are flanked by inversely orientated and closely spaced repeat sequences. Inter-simple sequence repeats are flanked by microsatellites that have identical sequences and are inversely orientated.

1. 8. 5. Restriction Fragment Length Polymorphism (RFLP)

In this technique DNA is digested with restriction enzymes known as restriction endonucleases (REs) which are isolated from bacteria and cut DNA at a constant position within a specific recognition sequence, typically 4-6bp long. (Dowling *et al.*, 1996) The resultant fragments are separated by gel electrophoresis and blotted onto a filter, then probes are hybridised to the DNA. RFLPs give highly reproducible patterns and are co-dominant markers. However they do have technical limitations including the need for a good supply of probes, blotting and hybridisation steps being time consuming and large quantities (10 μg per digestion) of good quality DNA are required (Karp *et al.*, 1996).

1. 8. 6. Amplified Fragment Length Polymorphism (AFLP)

This technique employs PCR to amplify a random assortment of DNA restriction fragments generated using two restriction enzymes. The basic AFLP protocol developed by Vos *et al.* (1995) can be separated into four sequential steps. During the first step DNA is isolated from the plant and digested with appropriate restriction endonucleases (RE). For most plant DNAs, two REs are used in the digestion – one a rare cutter having a 6bp recognition site and the other a frequent cutter with a 4bp recognition site. In the second step, specific double stranded (ds) adapters are ligated to the ends of the digested DNAs to generate chimeric molecules of known and unknown sequence. The third step is the PCR phase. The chimeric target DNA:adapter DNA fragments are first subjected to one or more rounds of PCR "preamplification" to ensure that the investigator has sufficient template DNA for fingerprinting PCRs. After preamplification the template DNAs are selectively PCR amplified using oligonucleotide primers that are homologous to the ds adapter and RE recognition site sequences but having extensions of one to three bases on their 3' ends (AFLP primers). During the final step of AFLP the PCR products are resolved on polyacrylamide sequencing gels and subsequently analysed for fingerprints of interest (Liscum, 1999). It is highly effective even in species whose populations are known to be in rapid decline, generating upwards of 50 variable genetic markers. The adequate characterisation of genetic diversity within rare and endangered species is often hampered by the difficulty of collecting large samples of individuals from a given population so the simultaneous amplification of large numbers of polymorphic DNA fragments represents perhaps the greatest advantage of the AFLP technique (Travis *et al.*, 1996). The technique requires no initial sequence information or characterisation of probes yet the results are comparable to RFLP studies but not as good at detecting heterozygotes, but they are technically more demanding and require more DNA (1μg per reaction) than RAPDs (Karp *et al.*, 1996). AFLPs can also be automated for use on as automated DNA sequencer (analyser), in this case primers are labelled with fluorescent dyes (Hodkinson *et al.*, 2002).

1. 8. 7. *DNA Sequencing*

The development of the polymerase chain reaction allowed direct sequencing from complex genomic DNA. Starting with virtually any amount of DNA, it is possible to amplify a target sequence up to microgram quantities. Double stranded DNA produced may be sequenced directly or by generating single stranded DNA from the amplification product, by treatment with exonuclease or by use of bitinylated primers. Sequences may be interpreted by reading them through autoradiographs or by sequence comparison and alignment. It is possible to sequence DNA from a wide variety of sources including preserved specimens and fossils (Hillis *et al.*, 1996). However, botanists may experience difficulties in detecting sequence variations below the species level (Karp *et al.*, 1996).

In using molecular techniques, it is important to recognise that all molecular diversity data are subject to experimental errors, which differ depending on the technique. PCR reactions using

single primers are more prone to irreproducibility than those using two primers; and so on. Reproducibility is the only easy way of assessing the quality of the data.

1. 9. *In vitro* propagation and somaclonal variation

It is well established that the process of tissue culture can generate both epigenetic and heritable variation (Larkin and Scowcroft, 1981; Jain, 2001). The genetic variability induced by *in vitro* conditions is known as somaclonal variation (Larkin and Scowcroft, 1981) and it is not limited to any particular group of plants. Chromosome instability and somaclonal variation are frequently associated with plant regeneration from unorganized callus producing adventitious buds rather than from axillary meristems (Piola *et al.*, 1999). However, in pineapple, cultivars are derived from somaclonal variants obtained by proliferation of dormant axillary buds (Soneji *et al.*, 2002).

Somaclonal variation is of practical interest due to its potential uses in plant breeding, but on the other hand, if clonal *in vitro* propagation or transformation is the main goal, it becomes an unwelcome phenomenon (Polanco and Ruiz, 2002). Thus it is important to know the frequency, the genomic distribution, the mechanisms and the factors influencing somaclonal variation, both from a theoretical and practical point of view (Polanco and Ruiz, 2002). The somaclonal variant frequency cannot be predicted and it depends on several factors (Feuser *et al.*, 2003). The plant species, explant type, and the donor genotype affect the variation rate. The composition of the culture medium, conditions of physical culture, and the duration between successive subcultures all affect the frequency of the variants (Vasil *et al.*, 1979; Vasil and Vasil, 1980; Ahuja, 1987).

The process of somaclonal variation is believed to require multiple genetic and/or epigenetic events which affect patterns of expression, or result in mutational alteration of genes (Leroy *et al.*, 2000a). Various molecular mechanisms may be responsible for the DNA mutation and genetic instability leading to the development of variations. Such molecular mechanisms include DNA damage and mutation, alteration of cell ability to repair damaged or mutated DNA, alteration of genes for cell-cycle control mechanisms, DNA methylation (Merlo *et al.*, 1995). However, only few mechanisms of somaclonal variation have been described in the literature. An individual degree of gene sensitivity to DNA damaging agents depends on factors such as the sequence of the gene, and whether it is actively transcribed or replicated (Markowitz *et al.*, 1995).

Various manifestations of patterns of genomic instability have been found in the human genome; several of them have been successfully used for the examination of human tumours. Microsatellite instability is one among these manifestations of genomic instability (Thibodeau *et al.*, 1993). It corresponds to an alteration in size of simple repeat sequences. A finding of microsatellite instability implies the presence of mutations in at least one gene involved in DNA mismatch repair mechanisms (Strand *et al.*, 1993). Thus, though a method to detect microsatellite length constitutes

an important tool in genetic characterisation of cultivars, it does not allow one to detect major forms of genomic instability such as molecular aneuploidy arising from deletions, amplifications, translocations, insertions, recombination and chemical alteration (Stoler *et al.*, 1999). Activation of transposons has also been shown to contribute to variation among tissue culture generated plants (Hirochika *et al.*, 1996; Pereza-Echeverria *et al.*, 2001; Phillips *et al.*, 1994).

Over the last few years several techniques for identifying genetic polymorphisms have been described. RFLP analysis was one of the first techniques widely used to detect variation at the sequence level (Botstein *et al.*, 1980). Later, PCR based techniques were developed, among them RAPD (Williams *et al.*, 1990), and its variants Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) (Welsh and McClelland, 1990), and DNA Amplification Fingerprinting (DAF) (Caetano-Anolles *et al.*, 1991). The more reproducible method of AFLP has also been used (Carolan *et al.*, 2002). The major drawback to these techniques is their sensitivity to DNA quality and reaction conditions (Leroy *et al.*, 2000a).

The use of molecular techniques as a means of evaluating the genetic stability of plants regenerated through tissue culture is very frequent nowadays because molecular markers can characterise somaclonal variation with greater precision and less effort than karyological and phenotypic analyses (Cloutier and Landry, 1994; Gonzalez *et al.*, 1996; Ruiz *et al.*, 1992). RAPD has proven affective to detect somaclonal variation in an increasing number of species, as demonstrated by recent studies of species such as rye (Linacero and Vazquez, 1992), date palm (Saker *et al.*, 2000) and garlic (Al-Zahim *et al.*, 1999). However, RAPD has been inconclusive in some cases. Fourre *et al.* (1997) indicated that the RAPD technique revealed no somaclonal variation despite the large sample of DNA and primers used and the important morphological and cytogenetical variation observed in the analysed embryogenic clones of *Picea abies* (L.) Karst. Munthali *et al.* (1996) analysed regenerated sugar beet plantlets using 5607 RAPD markers, of which only three were polymorphic (0.05%). Other recent studies have indicated no detection of somaclonal variants using the RAPD method on begonia (Bouman and De Klerk, 2001), oak (Wilhelm, 2000), oil palm (although the analysis consisted of up to 8900 markers) (Rival *et al.*, 1998), or *Pinus thunbergii* Parl. (Goto *et al.*, 1998). The AFLP technique (Vos *et al.*, 1995) allows the identification of a greater number of polymorphisms per primer pair than RFLP or RAPD analysis. However, only a few studies of somaclonal variation have been done using AFLP markers so far. These studies analysed regenerated plants of oak (no variation was detected in embryogenic cell lines) (Wilhelm, 2000), pecan trees (variation was detected in culture lines of somatic embryos and within pecan trees from the same culture line) (Vendrame *et al.*, 1999; Vendrame *et al.*, 2000), sugarcane (Arencibia *et al.*, 1999), *Papaver bracteatum* L. (Carolan *et al.*, 2002), and *Arabidopsis thaliana* (variation was detected in plants regenerated by organogenesis from roots) (Polanco and Ruiz, 2002).

SSRs provide one of the most reliable and reproducible marker systems (Chambers and MacAvoy, 2000; Smulders *et al.*, 2001) and have been used in studies to distinguish between *Populus nigra* and *P. deltoids* individuals, to produce specific fingerprints of commercial clones and to detect hybrid *Popular* in natural Italian populations (Fossati *et al.*, 2003). They have been used to assess genetic diversity (Kantety *et al.*, 1995), to detect polymorphism among accessions (Zietkiewicz *et al.*, 1994; Tsumara *et al.*, 1996; Nagaoka and Ogihara, 1997), and they have been mapped to their locations on plant chromosomes (Kojima *et al.*, 1998). SSR technology has been used to identify clones of the potato cultivar Russet Burbank (Coleman *et al.*, 2003) and to test the genetic integrity of wheat (*Triticum aestivum* L.) germplasm after long-term storage (Boerner *et al.*, 2000). The somatic stability in peach (*Prunus persica*) and olive (*Olea europaea*) was assayed, using three long-living accessions of 'Redhaven' peach of different origin and 2-4 centuries-old plants of olive. No variations were found in peach, whereas in olive rare intra-variety polymorphisms were observed in several of the 35 microsatellites analysed (Cipriani *et al.*, 2001).

Barrett *et al.* (1997) compared RAPD and microsatellite markers to assess the fidelity of shoots from the crown, epicormic shoots and *in vitro* propagated shoots of *Quercus petraea* (Matt.) Liebl. from the same mother tree. No variation was found using either technique but it was concluded that the microsatellite PCR technique is more reliable than RAPD because of its gene specificity, it can provide additional information on genome organisation, and SSRs give a better resolution of amplified fragments than RAPD. Zulini *et al.* (2003) investigated the phenotypic and genotypic variability of the grapevine cultivar 'Picolit' by means of ampelographic and ampelometric descriptors and the use of the molecular markers (microsatellites and AFLPs). All the methods used gave similar results in identifying plants which showed variations, including extra alleles. Rahman and Rajora (2001) detected microsatellite DNA somaclonal variation in *Populus tremuloides* micropropagated from organised vegetative buds. AFLP and random amplified microsatellite polymorphism (RAMP) were used to identify somaclonal variation in transgenic sugarcane (*Saccharum* hybrid) (Arencibia *et al.*, 1999).

Leroy *et al.* (2000) used ISSR techniques (discussed in section 1. 8. 4.) to detect somaclonal variation in cauliflower callus from the same mother plant they report that cloning and sequencing of the altered ISSR bands can be used to identify and map the altered regions/ genes of the genome in abnormal tissues. Devarumath *et al.* 2002 used RAPD, ISSR and RFLP markers to evaluate the genetic integrity of three mature, elite tea (*Camellia*) clones propagated *in vitro* from nodal explants. ISSR fingerprinting (12.8%) detected more polymorphic loci than RAPD (4.28%). This is another example demonstrating that organised meristems cultures are not always genetically true to type.

1. 10. Microsatellite markers for *Fraxinus excelsior*.

Brachet *et al.* (1999) and Lefort *et al.* (1999) have characterized microsatellite markers in *F. excelsior*. Brachet *et al.* (1999) report using a method proposed by Fisher *et al.* (1996) which enables the identification of microsatellite loci without the expense of library screening. This approach, called 5' anchored PCR, employs a primer containing microsatellite repeats followed by a degenerated anchor at its 5' end. The amplification produces two close and inverted microsatellites and the region between them. This microsatellite-rich PCR profile is cloned to yield a genomic library enriched for microsatellites. Lefort *et al.* (1999) produced microsatellite markers by creating a microsatellite-enriched library of *F. excelsior* using a method described by Edwards *et al.* (1996). The markers produced in these studies have been used to assess the genetic structure of *F. excelsior* populations in Bulgaria (Heuertz *et al.*, 2001), France (Morand *et al.*, 2002), Romania (Heuertz *et al.*, 2003) and Ireland (Harbourne, 2004). Morand-Prieur *et al.* (2003) investigated the polygamous breeding system of *F. excelsior* using nuclear microsatellite markers to assign the parentage of seedlings produced by controlled crosses. Nuclear microsatellite markers were recently used by Heuertz *et al.* (2004) to examine patterns of genetic structure between western and south eastern European populations of *F. excelsior* to gain insight into the postglacial recolonisation process.

Morand-Prieur *et al.* (2002) used RFLP, PCR-RFLP and microsatellite analysis to detect chloroplast polymorphisms between two ash species, *F. excelsior* and *F. angustifolia*. Morand *et al.* (2001) isolated mitochondrial DNA for use in restriction analysis from *Fraxinus* species including from *F. excelsior*. Jeandroz *et al.* (1996) used RAPD markers to distinguish between *F. excelsior* and *F. oxyphylla* and their putative hybrids. Raquin *et al.* (2002) used both RAPD and nuclear microsatellite markers to demonstrate hybridization between *F. excelsior* and *F. angustifolia*.

1. 11. Aims/ structure of thesis

In this thesis research was conducted using the most appropriate methods available to develop an improved protocol for all stages of the micropropagation cycle for *F. excelsior.*

Chapter two of this project aimed to develop a system of forcing stem segments containing dormant winter buds into growth by testing the effectiveness of heat treatments on breaking bud dormancy. Therefore, extending the propagation season by utilising the relatively free months of late winter/ early spring (see chapter 3), leaves from the forced stems can also be used for DNA extraction purposes (see chapter 6).

Chapter three aimed to develop a suitable protocol for the establishment of proliferating shoot cultures *in vitro* from buds of mature ash trees by optimising the explant selection process, sterilisation techniques and media composition including the use of plant growth regulators.

Chapter four aimed to optimise the shoot production stage of the micropropagation cycle by testing the culture regime, nutrient media salt composition, carbohydrate source and plant growth regulator concentration for a range of genotypes.

Chapter five aimed to elucidate a reproducible method of root induction suitable for a range of genotypes, this was achieved by testing auxin concentration and exposure time in the media, dark treatments, adding cytokinin to the media and using different nutrient media salt compositions. The survival of rooted and non-rooted shoots during weaning to the *ex vitro* environment was be tested, and the effect of cold treatment of weaned plants over winter on subsequent growth rates analysed. Further to experiments by Douglas (personal communication) showing improved rooting of tissue-culture produced ash plants of mature genotypes from summer stem cuttings, the rooting capacity of dormant stem cuttings from tissue-culture produced plants was tested.

Chapter six aimed to test the genotypic fidelity of vegetatively reproduced clones of the selected mature genotypes in the breeding collection and furthermore to analyse the genetic diversity of a sample of the breeding collection using microsatellite markers.

In chapter seven the key findings of this project are discussed and their relevance to the agronomic improvement of Irish ash for farm forestry highlighted.

CHAPTER 2: Winter dormancy breaking.

2. 1. Introduction

In most plant propagation systems, particularly involving deciduous trees, the growing season is confined to the spring/ summer seasons and the winter months are periods of relative inactivity. The establishment of a system where dormant stem segments are collected in the field and forced into growth under clean conditions in the greenhouse would enable culture initiation earlier in the season than if the buds were not forced. The benefits of which would include the extension of the propagation season from late winter through to early summer (using refrigerated dormant shoots) and giving plants an earlier start to growth. Attempts were made in this chapter to develop this system by experimenting with the use of heat treatments to break bud dormancy.

2. 2. Plant Dormancy

Dormancy in plants is defined as the temporary absence of visible growth (Romberger, 1963; Lang *et al.*, 1985; Lang, 1987; Lang *et al.*, 1987). Dormancy of trees and shrubs, particularly in temperate zones, has evolved as an adaptive strategy to survive the unfavourable environmental conditions such as drought in summer and frost in winter (Romberger, 1963; Powell, 1987; Borchert, 1991). In most woody plant species (including ash), dormancy is reflected through the rhythmic growth pattern (Romberger, 1963; Borchert, 1991; Crabbé and Barnola, 1996), i.e. an alternation of active and dormant phases. Even under continuously moist and warm equatorial climates, alternating phases between rapid shoot elongation and little or no growth are commonly observed (Hallé *et al.*, 1978; Crabbé and Barnola, 1996).

2. 2. 1. The stages of dormancy.

When seasonal shoot growth ceases, the plants first enter a reversible phase of inactivity called predormancy or quiescence (Romberger, 1963). Plants in this condition still have the capacity for growth, but the range of environmental conditions in which they can grow becomes narrower with the passage of time. The state of dormancy continues to deepen until true dormancy is reached and shoot apices cannot elongate even under the most favourable growing conditions. Eventually, true dormancy is terminated and a transition to post dormancy occurs. The tissues can again resume growth, at first under very narrow environmental limits and later under wider conditions. Finally the tissues are released from dormancy (Kramer Kozlowski, 1979; Vegis, 1964).

2. 2. 2. Regulation of growth

Following the breaking of dormancy, vegetative bud growth is associated with the action of specific hormones and is often accompanied by increased cell division (Horvath *et al.*, 2003). Cell division is represented by a continuous cycle of 'phases' (figure 2. 1.). Cells in G1 phase expand and prepare for

DNA replication, which occurs during S phase. After DNA replication, cells enter G2 phase and continue to expand and prepare for mitosis, which occurs during M phase (Horvath *et al.*, 2003). In plants, non-dividing cells can arrest at the G1–S-phase transition, just before DNA replication, or at the G2–M-phase transition, just before mitosis (den Boer and Murray, 2000). In most cases, cells in vegetative buds and shoots appear to be arrested in G1 phase, before the S phase of the cell cycle (Gutierrez *et al.*, 2002). Dormancy breaking results in upregulation of genes that act at the G1–S-phase transition, such as D-type cyclins (CYCD) and histones (Devitt and Stafstrom, 1995; Horvath *et al.*, 2002; Freeman *et al.*, 2003). In addition, several post-translational modifications to key enzymes involved in cell cycle regulation occur shortly after dormancy break in several plant systems (Campbell *et al.*, 1996; Horvath and Anderson, 2000). Consequently, the effort to understand the regulation of cell cycle genes is proving fruitful in understanding the molecular basis of growth arrest imposed by dormancy.

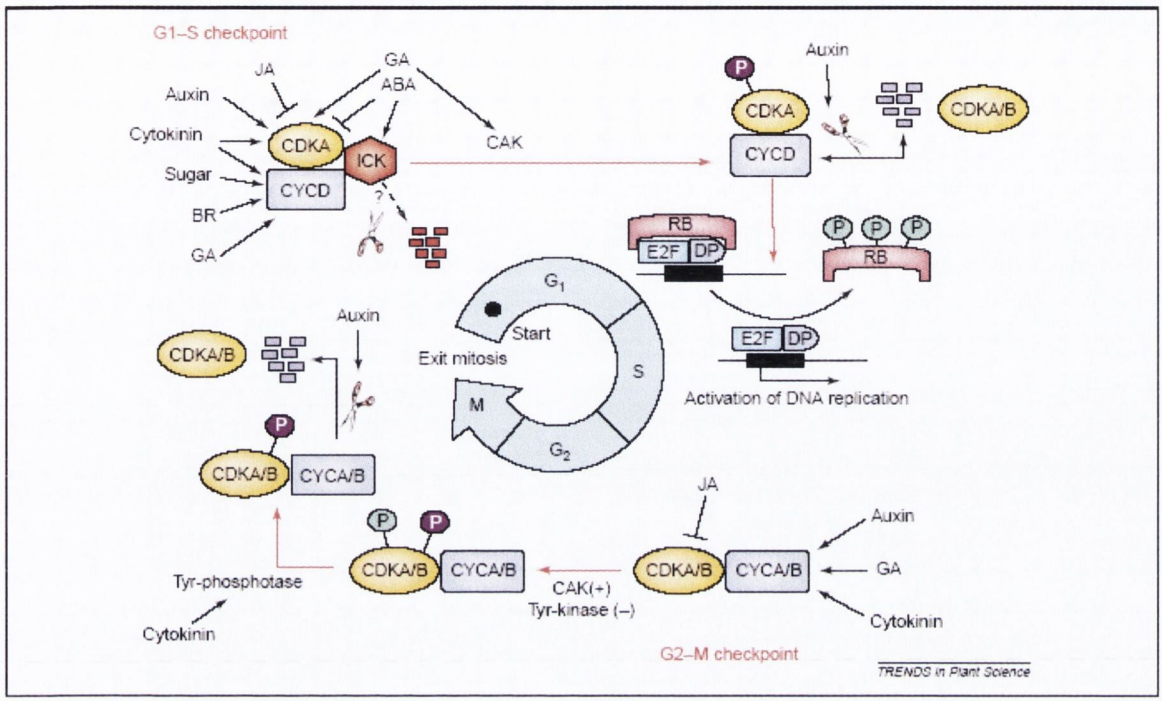


Figure 2. 1. Model for G1–S and G2–M transitions in plants based on combined models by Gutierrez *et al.* (2002), Anderson *et al.* (2002) and Stals and Inzé (2001) and on recent results obtained from plants and animals. Activation of G1 progression involves the expression of D-type cyclins (CYCD) and their catalytic subunit, cyclin-dependent kinase (CDKA), dissociation of CDK inhibitory protein (ICK1) from CDKA–CYCD complex, and phosphorylation of the Thr160 residue (P highlighted in purple) of CDKA. CYCD and CDKA are upregulated by various growth regulators including auxin, cytokinin, brassinosteroids (BR), sugar and gibberellic acid (GA). ICK1 is induced by abscisic acid (ABA). Phosphorylation of CDKA is the activity of CDK-activating kinase (CAK), which is induced by GA (Riou-Khamlichi, 2000). Active CDKA–CYCD complex hyperphosphorylates retinoblastoma protein (RB), which inhibits its binding to transcription factors (E2F) and the docking protein (DP), thus initiating chromatin remodeling, transcription activation, DNA replication and S-phase transition. The SCF (SKP1–Cullin–F-box-protein) complex mediates ubiquitination and proteolysis (scissors) of ICK1 (Henchoz *et al.*, 1997) and CYCD that is necessary to trigger the G1–S-phase transition in yeast (and possibly plants, denoted by '?'). Initiation of the G2–M-phase transition requires induction of the A- and B-type cyclins (CYCA/B) and the activity of A- and B-type CDKs (CDKA/B). The plant hormones auxin, cytokinin and GA have all been implicated in CYCA/B and CDKA/B expression and/or stability. At G2, the Thr160 (P highlighted in purple) of CDKs is positively phosphorylated (þ) by CAK, and the Thr14 or Tyr15 (P highlighted in green) of CDKs are negatively phosphorylated (2) by a tyrosine kinase in the CDKA/B–CYCA/B complex. A cytokinin-regulated tyrosine phosphatase (CDC25) removes the inhibitory phosphate and allows the G2–M-phase transition to occur. Commitment to mitosis requires ubiquitin dependent proteolysis of B-type cyclins. The anaphase-promoting complex (APC) regulates ubiquitination and proteolysis of CYCA/B. Auxin appears to be involved in the degradation of cyclins. Jasmonic acid (JA) inhibits CDK activity in both the G1–S-phase and the G2–M-phase transitions (taken from Horvath *et al.* 2003).

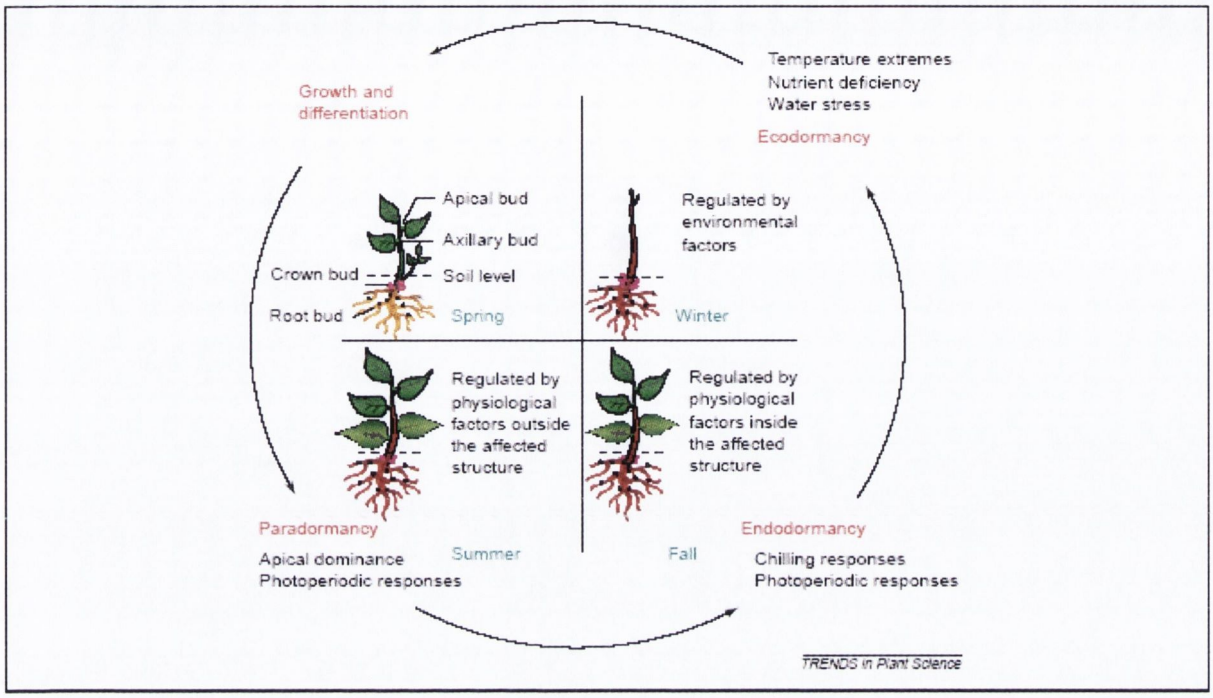


Figure 2. 2. Diagram of signals and typical seasons corresponding to the different types of dormancy. Dormancy is generally defined as the temporary suspension of visible growth of any plant structure (e.g. buds containing meristems). Lang *et al.* (1987) further categorized dormancy into paradormancy, endodormancy and ecodormancy. This figure illustrates the cycle of three types of dormancy and the general signals and seasons that are associated with dormancy of perennial weeds, woody plants (shrubs and trees) and shoot buds of tubers (potato and yam) (taken from Horvath *et al.* 2003).

2. 2. 3. Types of dormancy

Regrowth of new plant tissue from shoots was initially studied more than 100 years ago and it was noted that signals from the apical shoot inhibited growth of proximal buds. More recent studies have identified conditions under which bud growth is inhibited by signals generated inside the vegetative bud or as a direct result of unfavourable conditions for growth (Horvath *et al.*, 2003). Lang *et al.* 1987 categorized these seemingly separate growth inhibited or dormant states as: (i) paradormancy, the inhibition of growth by distal organs; (ii) endodormancy, the inhibition of growth by internal bud signals; and (iii) ecodormancy, the inhibition of growth by temporary or cyclic unfavourable environmental conditions such as low temperatures during winter (figure 2. 2.). Although these types of dormancy are usually thought of as occurring separately, any given bud might be simultaneously controlled by any, or all of the signals regulating these aspects of dormancy (Horvath *et al.*, 2003) (figure 2. 2.).

(1) Paradormancy

Paradormancy is the best studied of the three types of dormancy and is sometimes referred to as apical dominance or correlative inhibition. This process allows the plant to devote resources to reproduction and to control plant architecture; maximizing light harvesting while allowing for regeneration should the individual shoots become damaged (Horvath *et al.*, 2003). Many studies have implicated basipetally transported auxin as the primary signal regulating paradormancy, the active substance responsible for the inhibition of axillary buds was isolated from *Phaseolus* shoot tips and identified as IAA (Hillman, 1985). However, grafting studies have implicated other signals than auxin, produced in the roots and stem, as having a profound effect on shoot outgrowth (Beveridge *et al.*, 2000; Cline, 1994). The role of auxin in growth inhibition is complicated slightly by the apparent production of auxin in growing buds and by plant growth's requirement for auxin (Horvath *et al.*, 2003). However, auxin produced in distal meristems appears to affect the buds differently than does auxin produced inside the buds once they have broken dormancy (Cline, 1991). Auxin signaling inhibits the production or sensing of cytokinin, a plant hormone that is associated with axillary bud growth (Horvath *et al.*, 2003). The signaling mechanisms regulating the growth inhibitory effects of auxin on the buds are beginning to be understood. Several genes involved in the auxin-regulated growth inhibition have been identified in pea (*Pisum sativum*) and *Arabidopsis* (Beveridge *et al.*, 2003; Leyser, 2003).

Other plant hormones that act in apical dominance and plant growth include gibberellins and ABA. GA promotes growth, whereas ABA inhibits growth. Studies have indicated that M-phase progression in rice requires GA signaling (Sauter, 1997), and GA induces S-phase progression but not full induction and growth in paradormant underground buds of leafy spurge (Horvath *et al.*, 2002). Evidence suggests that ABA acts within axillary buds as a second factor whose level can be regulated by the dominant organ i. e. the apical bud (Tamas, 1995). The apical bud elicits high endogenous ABA levels

in quiescent axillary buds and the release of the buds from dominance is correlated with a decrease in ABA concentration in the buds (Tamas, 1995). However, these effects could not be confirmed in some studies (Pilate *et al.*, 1989), and in certain experiments on decapitation the outgrowth of axillary buds preceded, rather than followed, the decline in bud ABA levels (Knox and Wareing, 1984).

(2) Endodormancy

Endodormancy is the result of physiological changes internal to the bud that prevent untimely growth during seasonal transitions, when environmental conditions often fluctuate between those permissive or inhibitory to growth. Endodormancy provides an important mechanism for protecting vegetative buds by ensuring that meristems will not resume growth until the stable return of permissive conditions (Horvath *et al.*, 2003). In contrast to paradormancy, the molecular aspects of endodormancy are poorly understood however, the signals that induce or break endodormancy are reasonably well characterized (Horvath *et al.*, 2003).

Light and temperature both play a significant role in the induction and breaking of endodormancy, with light playing the dominant role in most woody perennials (Horvath *et al.*, 2003). In deciduous trees, shortening day length induces a developmental change in terminal buds that results in a leaf primordium forming scales instead of leaf buds (Okuba, 2000). In trees such as birch (*Betula papyrifera*) and poplar (*Populus tremuloides*), additional changes take place that induce cold hardiness, cessation of cell division and induction of dormancy in the terminal meristems (Li *et al.*, 2003). Extended chilling or freezing are often required to break the endodormant state, but chemicals such as hydrogen cyanamide (HC) break endodormancy as well (Henzell *et al.*, 1991). Although the mechanisms for dormancy release by HC are unknown, there is evidence that it requires the action of a sucrose non-fermenting (SNF) like protein kinase (Or *et al.*, 2000).

Some signals mediating the induction of endodormancy are being characterized, although the mechanisms through which the signals are mediated remain to be elucidated. Phytochrome has long been known to be a key regulator of light responses in plants. Quantitative trait locus (QTL) analysis of progeny between poplar ecotypes with differing dormancy induction thresholds identified two major QTLs, one of which mapped to a region of the chromosome that contains a phytochrome-encoding gene (Frewen *et al.*, 2000). Senescence in plants is often associated with, or occurs simultaneously with, induction of endodormancy. Ethylene and ABA play roles in the induction of plant senescence. In fact, senescence is implicated in the induction of endodormancy in several plant systems (Fedoroff, 2002). Ethylene directly induces endodormancy in potato microtubers (Suttle, 1998). Additionally, the role of ABA in endodormancy is well established in several systems, and instances of phytochrome acting synergistically with ethylene and ABA are known (Finlayson *et al.*, 1998; Weatherwax *et al.*,

1998). A better understanding of the molecular mechanism through which these signalling pathways operate is developing but, with the exception of ABA action, no solid connection has been characterized between the signalling pathways for these stimuli and dormancy (Horvath *et al.*, 2003). Several studies cast doubt on the view that hormones alone control the induction and breaking of bud dormancy and current evidence indicates that control of bud dormancy is very complex and involves interactive effects of growth hormones and other compounds (Kolzowski and Pallardy, 1997b).

Another approach has concentrated on the change in water status of the buds, Faust *et al.* (1991) showed that during dormancy, water in the buds is found in a more bound state, while towards release from dormancy it becomes freer. They suggested that the change in state of water controls induction of dormancy and later its release. More recent work failed to verify that hypothesis and indicated that the development of bound water in dormant buds is more closely connected to cold hardiness than with dormancy (Erez *et al.*, 1998). Erez (2002) suggests that the evolution of stress resistance at the level of membranes was adopted as part of the mechanism involved in the state of dormancy. The stress resistance mechanism for maintaining the functionality of membranes in a hostile environment involved desaturation of lipids and a change in membrane viscosity and hydration. The plant adapted the stress related development to the dormancy mechanism by using it in non-photosynthetic organs as a time-temperature measuring mechanism to obtain information on when the buds should respond to high temperatures in order to resume growth.

More recently, there has been an effort to characterize molecular changes directly regulated during onset and breaking of dormancy. The expression of several dormancy responsive genes in the buds of deciduous trees and other plants has been characterized (Horvath *et al.*, 2003). Perhaps the most interesting studies have focused on genes coding for KNOTTED like homeodomain proteins. Certain members of this gene family, along with members of the CLAVATA gene group and WUSCHEL, play a crucial role in the proliferation of undifferentiated cells of shoot meristems and are required for maintenance of growth (Clark *et al.*, 1996). The expression of KNOTTED-like homeobox protein 2 gene (KNAP2) is upregulated during dormancy onset but downregulated during breaking of dormancy in apple (Brunel *et al.*, 2002). In a related result, overexpression of a KNOTTED-like gene in potato reduced the level of GA in the whole plant (Rosin *et al.*, 2003). These findings are counter intuitive considering that KNOTTED-like genes are often associated with increased meristem growth. More consistent with the hypothesis that KNOTTED-like genes should be positively associated with growing rather than dormant meristems are studies in *Arabidopsis* indicating that a KNOTTED-like gene from *Arabidopsis thaliana* 2 (KNAT2) responds positively to cytokinin and antagonistically to ethylene signaling (Hamant *et al.*, 2002). Also, mutations in the PASTICCINO genes, which negatively regulate cell division by coupling hormonal control of cell proliferation and development, can partially

complement the meristemless phenotype associated with mutations in certain KNOTTED genes (Harrar *et al.*, 2003). It should be realized that KNOTTED-like genes are a small gene family. Thus, one explanation for these conflicting results is that different members of the KNOTTED-like homeodomain gene family might be expressed differently (Horvath *et al.*, 2003).

In addition to genes that are expressed differently, some proteins are relocated or modified during the induction and release of endodormancy. During dormancy onset in silver birch (*Betula pubescens*), the plasmodesmata are blocked by 1, 3-b-D-glucan. They remained so until chilling caused movement of small spherosome-like vacuoles containing 1,3-b-D-glucanase that aligned with the plasmodesmata before releasing 1,3-b-D-glucanase. Dyes and fluorescein-tagged GA confirmed that the reopening of the plasmodesmata followed chilling (Rinne *et al.*, 2001). Additionally, various isoforms of pectin methylesterase show different expression patterns during bud dormancy and release in hybrid aspen (Micheli *et al.*, 2000), and differences in the temperature dependent K_m of plasmalemma ATPase activity were noted in vegetative buds of peach (Aue *et al.*, 2000).

Along with changes in gene expression, there is also evidence for more general epigenetic changes associated with endodormancy induction and release. Changes in DNA methylation have been observed during the induction and breaking of endodormancy in potato buds (Law and Suttle, 2003). Increased DNA methylation following induction of dormancy suggests that chromatin remodeling might play a role in regulating bud dormancy (Horvath *et al.*, 2003). Interestingly, the previously mentioned SNF1-like protein kinase, activated in grape by hydrogen cyanamide, is similar to a known component of a DNA modifying protein complex SWI–SNF from yeast and animals (Fan *et al.*, 2003). Other components of this complex interact with RB–E2F (figure 2. 1.) in both plants and animals (Shen, 2002). Notably, flowering is also regulated by light and often requires extended chilling or freezing temperatures for induction of flowering. Recent advances in the molecular mechanisms regulating the temperature and light regulation of flowering have defined several proteins involved in chromatin remodeling (Horvath *et al.*, 2003). One such protein, fatty acid synthase (FAS) 1, is involved in chromosomal remodeling and the flowering response, and is regulated in a cell-cycle-dependent manner in cell cultures of *Arabidopsis* (Menges *et al.*, 2002). Given the overlap in the physical signals regulating flowering and endodormancy, it might prove fruitful to search for common signaling pathways as well (Horvath *et al.*, 2003).

Bergervoet *et al.* (1999) analysed changes in *beta*-tubulin levels and amounts of DNA following dormancy induction and release in Norway maple (*Acer platanoides* L.), apple (*Malus* '*M*9'), pedunculate oak (*Q. robur*), Scots pine (*Pinus sylvestris* L.) and rose (*Rosa corymbifera* 'Laxa'). Differences in nuclear DNA profiles and *beta*-tubulin levels were found between dormant and active

status with both apical and axillary buds of all five woody plant species. The results indicated that *beta*-tubulin accumulates upon dormancy release and decreases upon dormancy induction in axillary buds of the species studied. In tomato seeds, *beta*-tubulin accumulation and DNA replication were used as a marker to monitor the start of germination processes (de Castro *et al.*, 1995; Bino *et al.*, 1996). Results in the study by Bergervoet *et al.* (1999) demonstrated that detection of *beta*-tubulin accumulation may be applicable for monitoring dormancy induction and dormancy termination in tree buds.

(3) Ecodormancy

Ecodormancy is imposed by external environmental factors such as cold or drought stress, which induce critical signals that cause bud growth to stop and to prevent further bud growth. These extended periods of environmentally unfavourable growing conditions generally are required to signal the breaking of endodormancy, while at the same time imposing ecodormancy (Horvath *et al.*, 2003). Although the molecular mechanisms controlling ecodormancy have not been fully characterized, there is sufficient information on the physiological signal transduction processes regulating responses to cold and drought to provide one possible mechanism for growth inhibition (Horvath *et al.*, 2003). The plant hormone ABA has long been recognized as a key signal induced during both cold and drought stress (Gilmour and Thomashow, 1991). Given the role that this hormone probably plays in regulating cyclin-dependant kinase inhibitory protein (ICK1), which is involved in the activation of the G1 phase of the cell cycle, it is easy to envision a model in which cold or drought-induced ABA accumulation blocks further growth and development by turning on ICK1 to prevent cell division in buds (Horvath *et al.*, 2003) (figure 2. 1.).

2. 3. Breaking bud dormancy

In the temperate zone, bud dormancy is most commonly induced by low temperatures and broken by winter cold. Freezing temperatures are not required for breaking dormancy, and temperatures of between 2 and 7°C are most effective (Kolzowski, 1997). The duration of chilling required for the release of bud dormancy varies with plant species, genotype, and the location of buds on the plant, site and even with the weather of the previous season. The effects of low temperature on dormancy release vary with the depth of bud dormancy. When buds are in a state of deep dormancy, the range of low temperatures needed to break dormancy is narrow. In comparison, during late dormancy, when the buds have been exposed to some chilling, the temperature range for bud break widens (Kolzowski and Pallardy, 1997b).

A variety of near-lethal stresses are capable of abruptly releasing buds of woody plants from dormancy (Fuchigami and Nee, 1987). In particular, near-lethal heat stress is effective in releasing buds of woody plants from dormancy (Wang and Faust, 1994; Shirazi and Fuchigami, 1995; Wisniewski *et al.*, 1996).

Shirazi and Fuchigami (1993) suggested that stresses in general may be universally involved in the breaking of bud dormancy and that the impact of near-lethal stresses on bud dormancy may explain why chilling models used to predict the development of bud dormancy do not work satisfactorily in some environments.

Exposure to near lethal stresses in addition to heat, including, chemicals, freezing, light, drought, injury, toxic substances and anoxia are all capable of breaking dormancy in several woody plant species (Fuchigami and Nee, 1987; Shirazi and Fuchigami, 1995). The mechanism by which a short exposure to heat stress overrides dormancy in woody plants is poorly understood but nonetheless is effective in releasing plants from dormancy (Wisniewski *et al.*, 1997). Non lethal stress treatments that break bud dormancy cause electrolyte leakage from exposed tissues (Shirazi and Fuchigami, 1993; Wisniewski *et al.*, 1996), stimulate ethylene production (Nee, 1987) and cause an increase in antioxidants (Fuchigami and Nee, 1987). Siller-Cepeda *et al.* (1992) showed that both chilling and near lethal stresses caused an increase in glutathione concentration concurrent with the breaking of dormancy in peaches. Exposure to high temperatures generally results in an inhibition of normal protein synthesis and an increase in the translation of mRNAs for heat shock proteins (HSPs) (Gurley and Key, 1991). Increased degradation of proteins in response to heat shock is also common although the mechanism has not been well characterised (Ferguson *et al.*, 1994). Wisniewski *et al.* (1996) postulated that heat induced denaturation of regulatory proteins controlling dormancy may lead to the removal of intrinsic blocks preventing the growth of buds.

Wisniewski *et al.* (1997) characterised the ability of near lethal heat stress to overcome bud dormancy in hybrid poplar (*Populus nigra "Charkowiensis"* x *P. nigra* (L.) *"Incrassate"*) and examined the effects of this stress on the induction of heat stress proteins and ubiquitin. Their findings showed that near lethal heat stress (40-45 °C) induced bud break in dormant poplar and increased the rate of bud break during ecodormancy, indicating that the effect of the heat treatments was dependent on the stage of dormancy. Temperatures that stimulated bud break in winter were lethal in early spring, reflecting changes in the thermotolerance of the buds. Although induction of different classes of HSPs in bud tissues were observed at different times of the year, the induction of HSPs and ubiquitin was not associated with the temperature that was most effective in inducing bud break. Neither the ubiquitin response nor the early presence of HSPs was associated with the highest percentage bud break. If breakdown of regulatory proteins controlling dormancy is necessary for bud break to occur, then the breakdown does not involve ubiquitin; however, it may occur through non-specific leakage from cells as suggested by an increase in electrolyte leakage.

Shirazi and Fuchigami (1995) investigated the effects of near lethal heat stress applied to intact shoots of red osier dogwood (*Cornus sericea* L.) during early, deep and late dormancy. Their findings suggested that heat stress applied during early and late dormancy was most effective at breaking bud dormancy, however, only application during late dormancy resulted in normal stem elongation. Wisniewski *et al.* (1997) noted that the ability of near lethal heat stress to overcome dormancy was time (stage of dormancy) dependant in poplar (*Populus*). The response to heat stress applied during early or late dormancy was most pronounced. Thermotolerance increased during early dormancy then decreased during the latter part of late dormancy and these changes were reflected in the patterns of heat induced bud mortality and electrolyte leakage. The basis for the increase in thermotolerance is not known. Guy (1990) postulated that the accumulation of cold induced, stress related proteins plays a role. Robertson *et al.* (1994) reported the presence of proteins in cold acclimated cells suspension cultures of bromegrass (*Bromus inermis* Leyss) that conferred heat stability to extracts of total proteins from the cell cultures.

From the literature, it is clear that heat treatments can be effective in releasing buds from dormancy and are best applied near the end of dormancy when buds are in the ecodormant state. Attempts were made in this thesis to induce the early release from dormancy of ash buds attached to stem cuttings collected in March. Treatments involving exposure of the cuttings to heat with or without water contact and at various temperatures were tested.

2. 4. Materials and methods

A. Plant material

In February 2002 and 2003 dormant ash shoots consisting of the apical bud and 30 – 40 cm of stem were collected in north Co. Dublin, from hedgerows cut in the preceding spring. The dormant shoots were placed in plastic bags and stored at 4°C until use in experiments. Similar dormant shoots were collected from selected ash genotypes in a stock plantation of grafted plants at Teagasc, Kinsealy (refer to chapter 3 section 3. 7. A. (2)).

B. Preparation of material and experimental conditions

Stem cuttings were obtained from the dormant shoots by removal of the apical bud. The stem cuttings consisted of two pairs of sub-apical buds with the basal cut made 1cm below the basal node (figure 2. 3, step 1) (Silveira and Cottignies, 1994). All heat treatment of the stem cuttings was administered in the water bath (figure 2. 3, step 2). Controls received no heat or water treatment and remained in the fridge at 4°C until removed to the glasshouse with the heat treated stems. Immediately after treatments, the stem cuttings were planted at 5cm intervals, in seed trays containing a substrate of 3-

4cm deep peat–vermiculite-perlite (v/v/v, 2:2:1). The basal 30mm portion of each stem cutting, including one pair of axillary buds, was immersed in the substrate. Seed trays were then placed into a larger tray, which acted as a water reservoir so the bases of the cuttings were wet (figure 2. 3, step 3), (Silveira and Cottignies, 1994). The stem cuttings were placed in the greenhouse for forcing under Philips SON 400W lights (170 μmol m^{-2} s^{-1} at bench level) with a 16-hour photoperiod, temperature in the greenhouse ranged from 15-22°C. In 2003 there was no heating provided in the greenhouse and temperatures ranged from 9-29 °C.

C. Data collection and analysis

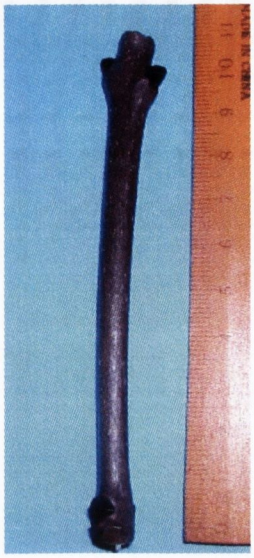
Each treatment was replicated six times with four individuals per replicate in a randomised block design. Recordings were made of days to bud burst defined as the number of days after treatment until the emergence of the first leaves (figure 2. 3, step 4). Percentage bud burst (the percentage of shoots in each treatment on which the buds has burst) was recorded after three weeks, by which time all buds were expected to have burst. Analysis of variance (ANOVA) was performed using the Statistica 6.0 statistical package (StatSoft, Inc., 2001). Percentage bud burst data was arc sin transformed for analysis by One-way ANOVA, the arc sin transformation achieves constant variances for the observations and makes analysis of the transformed data within the ANOVA model possible. Mean separation was performed using Tukey's honest significant difference (HSD) test. Significance was recorded at $p \leq 0.05$.

D. Experimental treatments

Experiments were performed on the stem cuttings to investigate the effectiveness of heat treatments and forcing in releasing the buds from dormancy.

(1) The effect of heat treatment with and without water contact on dormancy breaking.

To investigate the effect of heat treatment, with and without water contact, on breaking dormancy of ash buds, treatments of 38°C for 12 hours (Silveira and Cottignies, 1994) were administered to stem cuttings of hedgerow collected material in March and April 2002. The stem cuttings were divided randomly into three groups, the first received no heat or water treatment (the control group), the second were treated in the water bath enclosed in water tight plastic bags and the third group was immersed in the water bath in perforated plastic bags.



Step 1. Stem cutting.

Step 2. Stems were heat treated in the water bath.

Step 3. During forcing in the greenhouse, stems were placed in a seed tray with a water reservoir.

Step 4. Bud burst, defined as the emergence of the first leaves.

Figure 2. 3. A flow diagram showing the steps involved in heat treatment of the stem cuttings and forcing in the greenhouse.

(2) The effect of one-hour treatments in direct water contact at temperatures ranging from 23-52°C on breaking dormancy.

To determine the optimal temperature to induce early bud burst in a shorter water treatment period than the 12 hour treatment described above, one-hour treatments at temperatures of 23°C, 28°C, 32°C, 37°C, 42°C, 47 °C and 52°C were administered by placing stem cuttings from hedgerow material directly in the water bath. This was done in March 2002 and 2003. Control stems were immersed in the water bath at ambient temperature (approximately 20°C) for one hour to eliminate the possibility that the heat effect was due solely to saturation of the tissues with water.

(3) The effect of heat treatment in direct water contact on breaking dormancy in different genotypes of ash.

Four genotypes of selected material (5, 47, 59 and 69) were treated at 37°C in direct water contact for one hour to investigate any genotype effect on breaking dormancy in March 2003.

2. 5. Results

(1) The effect of heat treatment, with and without water contact, on dormancy breaking.

Heat treatments (38°C for 12 hours) with or without water contact were administered to stem cuttings of hedgerow collected material in March and April 2002 (materials and methods section 2. 4. (1)) In both months of 2002, administering a heat treatment to stem cuttings of dormant winter buds in direct contact with water proved significantly more effective in releasing buds from dormancy than heat treatments administered with the stem cuttings placed in watertight bags or to controls given no heat treatment (table 2. 1.).

Table 2. 1. The effect of heat treatment (12 hours at 38°C) given with or without water contact on bud burst in dormant ash buds.

Treatment	*Days to bud burst ± S.E.* March 2002*	*Bud burst (%) March 2002*	*Days to bud burst ± S.E. April 2002*	*Bud burst (%) April 2002*
Control (no heat or water)	23.8 ± 1 [a]**	33.3[a]	23.3 ± 1.1 [a]	26.1[a]
Heat, without water contact	22.7 ± 0.9[a]	50.0[a]	23.0 ± 3.4 [a]	20.1[a]
Heat with water contact	15.0 ± 0[b]	95.8[b]	12.2 ± 3.7 [b]	79.1[b]

* ± Standard Error. **Different letters within columns indicate a significant difference according to Tukey's HSD ($p<0.0005$). Example: In the first results column 23.8 and 22.7 both followed by the letter a are not significantly different from each other as they carry the same letter but they are significantly different from 15.0 which is followed by the letter b.

In March 2002, the number of days to bud burst was significantly reduced by nine days in buds given heat and water treatment compared to controls and a reduction of eight days in buds given heat treatment with no water contact (One-way ANOVA, $p<0.00001$). Percentage bud burst was significantly higher in buds given heat treatment in direct water contact compared to the controls and buds given heat treatment without water contact (One-way ANOVA, $p<0.00002$) (table 2. 1.).

When the experiment was repeated in April 2002, the overall capacity for bud burst was lower than the previous month. The number of days to bud burst was reduced by eleven days in heat and water treated buds compared to the control buds and buds treated with heat and no water contact (One-way ANOVA, $p<0.00001$) (table 2. 1.). Percentage bud burst was significantly higher in buds given heat treatment in direct water contact compared to the controls and buds given heat treatment without water contact (One-way ANOVA, $p<0.00002$) (table 2. 1.). In both months there was no significant difference in days to bud burst or percentage bud burst between the control and buds that received heat treatment in plastic bags with no direct water contact (table 2. 1).

(2) The effect of one-hour treatments in direct water contact at temperatures ranging from 23-52°C on breaking dormancy.

To determine the optimal temperature to induce early bud burst, the effect of a range of temperatures (one-hour treatments at 23 - 52°C in water) were tested over two seasons in March 2002 and 2003.

Table 2. 2. The effect of temperature treatments given in water for one hour on bud burst of dormant winter buds in March 2002 and 2003.

Temperature (°C)	*Days to bud burst ± S.E. * March 2002*	*Bud burst (%) March 2002*	*Days to bud burst ± S.E. March 2003*	*Bud burst (%) March 2003*
Control	23.4±1 [a]**	33.3 [a]	25.4±0.8[a]	20.8[a]
23 °C	18.6±0.6[b]	87.5[b]	21.6±0.8[bc]	75[bc]
28 °C	17.8±0.6[bc]	91.7[b]	19.7±0.5[cd]	87.5[b]
32 °C	16.9±0.4[bc]	95.8[b]	19.4±0.5[d]	91.7[b]
37 °C	16.1±0.4[c]	91.7[b]	20.5±0.4[bcd]	93.3[b]
42 °C	17.7±0.5[bc]	100[b]	19.2±0.5[d]	95.8[b]
47 °C	17.6±0.4[bc]	95.8[b]	22±1.1[b]	45.8[ac]
***52 °C	N/ A	0	N/ A	0

* ± Standard error. **Different letters within columns indicate a significant difference according to Tukey's HSD (Days to bud burst: March 2002, $p<0.0005$, March 2003 $p<0.03$) (Percentage bud burst: March 2002: $p<0.00003$ and March 2003: $p<0.003$, except [32°C, 47°C] where $p<0.03$). *** All buds died

In both years, treating dormant stem cuttings at elevated temperatures reduced the number of days to bud break significantly compared to the controls (One-way ANOVA, $p<0.0001$), all stems treated at 52°C died and were excluded from the analysis. For stem cuttings treated in 2002, the temperature of 37°C accelerated bud burst by eight days compared to the control, while in 2003 the temperatures of 32°C and 42°C gave the greatest reduction in the number of days required for bud burst (table 2. 2, figure 2. 4). The percentage of stem cuttings showing bud burst was over 75% for each temperature of 23°C to 42°C in both years, control stems showed 33.3% and 20.8% bud burst in 2002 and 2003 respectively (table 2. 2).

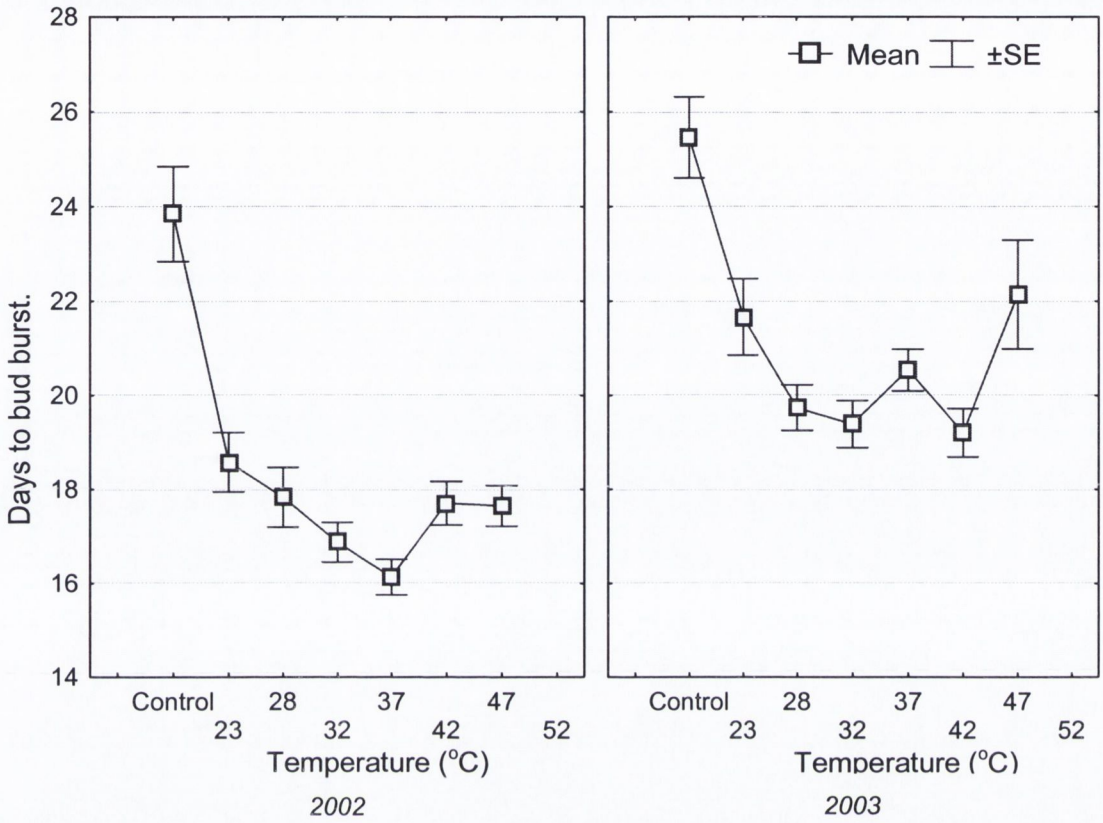


Figure 2. 4. Days to bud burst of dormant winter buds treated for one hour in direct water contact at temperatures ranging from 23°C to 52°C, and forced in the greenhouse, March 2002 and 2003.

(3) The effect of heat treatment on breaking dormancy in different genotypes of ash.

A heat treatment of 37°C for one hour was chosen to test the influence of the genotype on breaking bud dormancy in March 2003. Of the four selected genotypes (5, 47, 59 and 69) given heat treatment genotype 69 responded best to the treatment taking an average of 15 days to break bud and showing 100% bud burst at three weeks. Genotype 59 displayed a poor response to the treatment taking over 22 days to break bud and having approximately 40% bud burst at three weeks (table 2. 3).

Table 2. 3. The effect of heat treatment (37°C for one hour) on bud burst of dormant winter buds of different genotype in March 2003.

Genotype	*Days to bud burst ± S.E.**	*Bud burst (%)*
7	19.25±0.2[a]**	95.8[a]
47	20±0.5[a]	83.3[a]
59	22.5±0.6[b]	41.6[b]
69	15.0±0.4[c]	100[a]

* ± Standard error. **Different letters within columns indicate a significant difference according to Tukey's HSD ($p<0.0004$)

2. 6. Discussion

The aim of the dormancy breaking experiments was to identify the optimum treatments to break bud dormancy in stem cuttings of dormant winter buds of ash. The establishment of a system where dormant stem segments are collected in the field, heat treated and forced under clean conditions in the greenhouse enabled clean explants to be harvested for initiation earlier in the season than if the buds were not forced.

Warm water treatments have been used in the past to free plants from pathogens (Langens-Gerrits *et al.*, 1998) and to break dormancy (Vegis, 1932; Krasnosselskaya and Richter, 1942; Chouard, 1951; Silveira and Cottignies, 1994; Wisniewski *et al.*, 1996; Wisniewski *et al.*, 1997). The advantages of using temperature treatments are that there are no chemical residues and also that many endogenous pathogens are killed (Langens-Gerrits *et al.*, 1998). However, warm water treatment may damage the host plant (Baker, 1962a), the resistance of plant tissue depends on the physiological condition, for example size, moisture content, vigour, condition of external layers, temperature conditions during growth, dormancy level, age and genetic constitution (Baker, 1962a).

The results of the first set of experiments (section 2. 5 (1)) confirm the findings of Silveira and Cottignies (1994) that ash stem segments harvested during dormancy can sprout and produce young stems when a warm water dormancy breaking treatment is applied. The results in this chapter show that stem cuttings harvested during dormancy, and stored at 4°C for one month, can be induced to break dormancy by forcing under lights in a greenhouse, and that this may be accelerated by immersing the stems and buds in warm water (table 2. 1), a practice recommended for forcing woody plants (Vegis, 1932; Krasnosselskaya and Richter, 1942; Chouard, 1951). Storing the stem segments at 4°C prior to warm water treatment did not adversely affect bud burst (95.8% in March and 79.1% in April) when compared to the study by Silveira and Cottignies (1994) where stems were warm water

treated immediately after harvest and showed between 96% (January) and 76% (March) bud burst. Dormant shoots of *Malus x domestica* Borkh., *Castanea* spp. and *Fagus sylvatica* L. have been harvested and kept in cold storage prior to forcing in the laboratory (Skirvin *et al.*, 1986; Vieitez *et al.*, 1986; Chalupa, 1996).

Heat treatment alone (without water contact) did not accelerate bud burst (table 2. 1) and later experiments excluded the possibility the heat effect was due solely to the saturation of tissues with water (table 2. 2). These results concur with those of dormancy breaking heat treatments on poplar (*Populus nigra "Charkowiensis"* x *P. nigra "Incrassata"*) (Wisniewski *et al.*, 1997). It was noted by Wisniewski *et al.* (1997) that in preliminary experiments significantly more buds were released from dormancy when heat treatments were carried out in water using perforated plastic bags than in waterproof bags.

The results show that a one hour treatment in water at a range of temperatures is effective in releasing the buds from dormancy and that the ability of warm water treatment to overcome dormancy is temperature dependant (table 2. 2, figure 2. 4). This phenomenon has also been reported by other researchers working on woody plants (Boresch, 1928; Wisniewski *et al.*, 1994; Wisniewski *et al.*, 1997). The effectiveness of winter oil sprays that can be applied to break dormancy is dependant on bud temperature following application (Samish, 1954). Wisniewski *et al.* (1994; 1997) found that thermotolerance in hybrid poplar increased during endodormancy (October to January) and decreased during the latter part of ecodormancy (March). They report that as the season progressed and the buds were no longer endodormant, temperatures which had previously induced the highest percentage bud break (40 - 45°C), were resulting in inhibition of bud break and high bud mortality indicating a shift in the thermotolerance of the buds. The lower bud break recorded in April 2002 compared to March 2002 may be related to this mortality effect. The pattern of temperature induced mortality and inhibition of bud break can also be seen with the ash buds in March 2002 and 2003 where the percentage bud burst increased with temperature from the controls to the 42°C treatment, then at 47°C decreased marginally in 2002 and dramatically in 2003 (table 2. 2.). The number of days to bud burst shows an interesting pattern in that the buds take a shorter time to break with increasing temperature up until 42°C in 2002 and 47°C in 2003 after these points the buds take a longer time to break (almost two days longer in 2002 and three days in 2003), indicating that there may be inhibition of dormancy release at these temperatures. The differences in results between the two years may have resulted from the lack of heating in the greenhouse during forcing in 2003. The basis for change in thermotolerance in dormant buds is not known. Guy (1990) postulated that the accumulation of cold induced, stress related proteins plays a role. Robertson *et al.* (1994) reported the presence of proteins

in cold acclimated cells suspension cultures of bromegrass (*Bromus inermis* Leyss) that conferred heat stability to extracts of total proteins from the cell cultures.

The four different genotypes of ash tested showed different responses to the heat treatment and forcing (table 2. 3). Genotypes from different provenances may have been in varying stages of dormancy (Parmentier *et al.*, 1998) or genetic differences could have made the buds more or less susceptible to the heat treatment (Langens-Gerrits *et al.*, 1998). Genotype effects similar to those reported here have also been reported in other species including blueberry (*Vacinnium*) (Parmentier *et al.*, 1998).

The mechanism by which exposure to warm water treatments (and other near lethal stresses) break dormancy is poorly understood. Exposure to near lethal stresses results in increased electrolyte leakage from exposed tissues (Shirazi and Fuchigami, 1993; Wisniewski *et al.*, 1997) and an increase in antioxidants (Fuchigami and Nee, 1987; Siller-Cepeda *et al.*, 1992; Shirazi *et al.*, 1994; Wang and Faust, 1994). Dormancy breaking treatments, including pruning (Samish, 1954), cyanamide (H_2CN_2) (Shulman *et al.*, 1983), warm water treatment and mineral oils (Samish, 1954) have also been shown to increase respiration in the affected organ.

Wisniewski *et al.* (1997) studied the effects of near-lethal heat stress on bud break, HSPs and ubiquitin in dormant poplar. Neither the ubiquitin response nor the presence of HSPs was associated with the highest percentage bud break and it was concluded that if the breakdown of regulatory proteins controlling dormancy is necessary for bud break to occur then this breakdown does not involve ubiquitin; however it may occur through non-specific leakage from cells as suggested by an increase in electrolyte leakage. Both Wang and Faust (1990) working on apple (*Malus*), and Erez *et al.* (1997) working on peach (*Prunus*) found that as long as levels of the fatty acid linoleate found in the cell membranes are kept low, buds will remain dormant, but under high temperatures linoleate is desaturated to linolenate (Thompson, 1979) and its final level dictates bud break. Numerous studies have found an association with bud break, induced in paradormant or endodormant buds, by cold, heat or chemical means, and free radical removal through activated peroxide scavenging systems (Rowland and Arora, 1997). Wang and Faust (1988) treated vegetative apple buds with a cytokinin-type growth regulator, called TDZ and found this treatment diminished free radical formation and induced bud break, thus concluding that the removal of free radicals is associated with bud break. However, no causal relationship has been established between free radical removal and bud break (Rowland and Arora, 1997). Wang and Faust (1994) found an increase in the antioxidants ascorbate and glutathione in paradormant and endodormant apple buds induced to break with chilling treatment, near-lethal heat treatment, and treatment with the sulphur-containing compound allyl disulphide. Siller-Cepeda *et al.*

(1992) reported an increase in reduced glutathione (GSH) in peach buds during endodormancy, reaching a peak with satisfaction of the chilling requirement. They also reported an increase in GSH within 12 hours of treatment with cyanamide, followed by a large increase within 24 hours. Shirazi *et al.* (1994) report an increase in GSH in red-osier dogwood (*Cornus sericea* L.) with near-lethal heat stress. Collectively, these studies indicate that bud break, whether induced in paradormant or endodormant buds by cold, heat, or chemical means, is associated with free radical removal through activated peroxide-scavenging systems. Whether free radical removal is a prerequisite for resumption of growth or occurs simultaneously is not clear (Rowland and Arora, 1997).

In the search for the mechanism of dormancy control in buds, some researchers have focused on studying the changes in water status, using nuclear magnetic resonance (NMR) or magnetic resonance imaging (MRI) techniques (Parmentier *et al.*, 1998; Erez *et al.*, 1998; Faust *et al.*, 1991; Rowland *et al.*, 1992). These techniques detect T1 or T2 proton relaxation times of water. A short relaxation time is interpreted as an indication of bound water (water associated with macromolecules) and a longer relaxation time is indicative of freer water. Because the biological activity of tissues depends on the physiological state of water, changes in the mobility of water are expected to accompany or precede the physiological changes that occur in the buds of woody plants during dormancy induction and release (Parmentier *et al.*, 1998). Faust *et al.* (1991) with apple buds and Rowland *et al.* (1992) with blueberry flower buds reported that the state of water significantly changes with the development of endodormancy. Bound water is associated with the dormant state and water is gradually freed during the dormant period (Faust *et al.*, 1995), and rapidly converted to free water when resumption of growth is triggered by TDZ (Liu *et al*, 1993) or by forcing conditions. The results described in this chapter, showed that warm water treatments are more effective when the buds are in direct water contact, this may suggest that immersion in warm water hydrates the buds allowing the resumption of growth.

At the start of spring, plants require water for optimal hydrolysis of food storage macromolecules and for increased enzyme activity in order to resume activities that ceased during the winter dormancy (de Fay *et al.*, 2000). The water content of buds of maple (*Acer sp.*), alder (*Alnus sp.*), beech (*Fagus sp.*) and ash increases during the spring (Essiamah and Eschrich, 1986) and is correlated with the phenological state of buds in walnut (*Juglans sp.*) (Ameglio, 1994). In ash trees, the increase in osmotic and water potentials of buds at the end of the dormant period suggests that some water is available more than one week prior to the increase in bud water content and growth (Cottignies, 1983; 1990). A change in the state of water from a bound form in dormant buds to a freer form was detected shortly before bud burst in ash (Cottignies, 1990) and at the end of the chilling requirement in fruit trees (Faust *et al.*, 1991; Faust *et al.*, 1995; Rowland *et al.*, 1992). As for the mechanism of initial entry of

water into the embryonic shoots in early spring, Cottignies (1986) proposed that, in the case of ash, some water which was bound is liberated in the cells of the shoot apex and contributes to the hydrolysis of starch into soluble sugars, thus modifying the osmotic potential and therefore the requirement for water.

With regard to the use of warm water treatments to break bud dormancy, Boresch (1928) suggested that the effectiveness of water immersion depends on anaerobic conditions as well as water temperature and that the products of anaerobiosis are responsible for breaking dormancy. However, Vegis (1932) expressed doubt that dormancy release due to water immersion was a result of anaerobic respiration. Erez *et al.* (1980) showed that low oxygen atmospheres stimulate dormant peach (*Prunus persica* L.) buds to grow and this supported the long published findings by Boresch (1928) that anaerobic conditions will release buds from dormancy. Winter oil sprays that break dormancy may also act by restricting oxygen diffusion to the buds (Samish, 1954).

This study has shown that warm water dormancy breaking treatments can be applied to stem segments, from mature ash trees, that were harvested and stored at 4°C during dormancy. The recommended treatment to break dormancy is a one-hour soak in water at 37°C, followed by forcing in a heated greenhouse. The new shoots produced are grown in a clean environment and have been used to provide explants for *in vitro* culture (chapter 3) and leaves for DNA extraction (chapter 6).

CHAPTER 3: Establishing shoot cultures *in vitro*

3. 1. Introduction

The primary goal of the initiation stage in plant tissue culture is to establish an aseptic culture of the selected plant material (George, 1993). However, a major problem associated with woody plant culture is contamination of the explants (Skirvin *et al.*, 1986; Chalupa, 1996; Hammatt, 1997). In principle the methods for used for explant sterilisation and culture initiation should be reproducible, achieving the viability of every bud cultured is not essential because proliferating shoot cultures in the micropropagation phase can be achieved from a single viable bud. A compromise must be reached between an adequate sterilisation procedure and a reasonable rate of survival of non-contaminated explants. In order to achieve this task, certain areas of the initiation stage must be optimised; this includes the explant selection, sterilisation techniques, media composition and plant growth regulators.

3. 2. The initiation of mature *F. excelsior* explants *in vitro*

Regardless of the difficulties involved in establishing cultures from mature trees it has been achieved for *F. excelsior* in the past (Hammatt, 1994; Hammatt, 1997; Pierik and Sprenkels, 1997). Ash cultures have been initiated from dormant winter buds which were given heat treatment to break dormancy (Silveira and Cottignies, 1994), similarly Perez-Parron *et al.* (1994) initiated apical and nodal explants from mature *F. angustifolia* by inducing bud break from dormant stem segments *ex vitro*. Other authors working with mature trees of *F. excelsior* and related species used apical and nodal explants from naturally flushing shoots (Arrillaga *et al.*, 1992; Hammatt, 1994; Chalupa, 1990). The sterilants used for decontamination of *Fraxinus* shoots include ethanol (Arrillaga *et al.*, 1992; Hammatt, 1997; Pierik and Sprenkels; 1997), mercuric chloride (Chalupa, 1990), mercurobutol (Silveira and Cottignies, 1994), sodium hypochloride (Arrillaga *et al.*, 1992); Hammatt, 1994; Perez-Parron *et al.*, 1994), and calcium hypochlorite (Silveira and Cottignies, 1994) all the authors mentioned recommend to rinse the explants three times with sterile water after the use of sterilising solutions. Arrillaga *et al.* (1992) rinsed the explants of mature *F. ornus* under running water for 12 hours prior to sterilisation, a practice also recommended by Bellarosa (1989). To avoid browning of the explants Arrillaga *et al.* (1992) recommends placing explants onto a preconditioning medium containing 3% sucrose and agar. Perez-Parron *et al.* (1994) placed the initial explants onto a low salt basal medium containing Quoirin and Lepoivre (1977) (QL) salts and Linsmaier and Skoog (1965) vitamins. Hammatt (1994) placed explants from mature *F. excelsior* onto Driver and Kuniyuki (1984) basal medium. Silveira and Cottignies (1994) tested three media including MS, QL and woody plant medium (WPM) (Lloyd and McCown, 1981) and found that the low salt media WPM and QL were best suited for initiation of *F. excelsior* shoots *in vitro*. From the available literature, the use of plant growth regulators in the initiation medium does not appear necessary for culture establishment and is only incorporated in the shoot production phase of the micropropagation cycle for *F. excelsior* (Hammatt, 1994; Silveira and Cottignies, 1994).

3. 3. Challenges associated with culture initiation from mature trees

In vitro shoot culture has become one of the most common methods for tissue culture of woody plants. Explants taken from juvenile tissues, particularly from seedlings, are usually highly responsive, those taken from mature tissues can be recalcitrant *in vitro* and more difficult to decontaminate (San-Jose *et al.*, 1988, 1990; Arrillaga *et al.*, 1992; Perez-Parron *et al.*, 1994; Hammatt, 1994). The major problem initiating cultures from mature trees is the heavy contamination, particularly when material is collected from field-grown plants (Ruigini, 1986; Vieitez *et al.*, 1986; Hammatt, 1994; Chalupa, 1996). In many tree species the use of tissue culture techniques has been confined mainly to juvenile material. This limits the application of tissue culture as an alternative to traditional methods because most economically important traits are expressed only in mature trees, and juvenile –mature correlations are often low, plants can exhibit substantial differences in several morphological and physiological attributes, including leaf morphology (Kolzowski and Pallardy, 1997) and capacity to flower (Greenwood and Hutchison, 1993). The ease of propagation of many trees diminishes as they approach a size sufficient to allow reliable evaluation of their crop potential (Bonga, 1987), and in micropropagation it is more difficult to induce organogenesis and embryogenesis in explants from mature trees than from juvenile plant material (Bonja and von Aderkas, 1993; Quraishi *et al.*, 2004). The transition from juvenile to adult often occurs gradually, and some parts of an otherwise adult tree may retain juvenile characteristics for many years (Ballester *et al.*, 1990; Quraishi *et al.*, 2004). For vegetative propagation of forest trees by cuttings or by *in vitro* tissue culture, the most juvenile material available should be used, e.g. root suckers, stump sprouts, basal shoots or lower branches (Meier-Dinkel and Kleinschmit, 1990; Quraishi *et al.*, 2004). However, adult trees do not always have material with juvenile properties, and in such cases adult tissue must be rejuvenated in some way to allow its establishment *in vitro*. Pre-culture rejuvenation methods include severe pruning, spraying with cytokinins or gibberellins (Magalewski and Hackett, 1979; Bouriquet *et al.*, 1984), serial rooting or grafting on juvenile rootstocks (Franclet, 1983). Serial subculture of explants from mature trees (Gupta *et al.*, 1983) also results in a gradual increase in juvenility. The rejuvenating effect through *in vitro* propagation techniques has been described for several woody plant species including *Vitis vinifera* L. (Mullins *et al.*, 1979), mature hybrids of *Betula platyphylla* var. *japonica* x *B. pendula* (Meier-Dinkel, 1991), *Quercus petraea* (San-Jose *et al.*, 1990) and *F. excelsior* (Hammatt, 1994; Pierik and Sprenkels, 1997). Both Hammatt (1994) and Pierik and Sprenkels (1997) found that with successive subculturing shoots from mature ash clones became progressively easier to root.

Explants from adult trees often produce more phenolic substances (which blacken the medium), than juvenile ones (Quraishi *et al.*, 2004). Oxidation induced browning and production of brown exudates by explants often result in rapid death of the plant tissues and is one of the main problems that must be overcome when culturing woody plants (Wanstreet, 1982; Bellarosa, 1989; Manzanera *et al.*, 1996; Herman, 2000). Normally, it is difficult to protect plant segments from

oxidation and necrosis during explant excision (Nomura *et al.*, 1998). Even when explants seem healthy at the beginning of manipulation, they may turn brown and die at a later stage of culture. Browning can sometimes be reduced or avoided by growing plants under special conditions (Nomura *et al.*, 1998). For example, slightly etiolated plants grown under shade are better than those in full light, however it is difficult to control physiological conditions of source plants in the field (Nomura *et al.*, 1998). Placing cultures in darkness for a day or two after subculturing can reduce browning in roses (Pittet and Moncousin, 1982). However, in callus cultures of *Quercus robur* no stimulating or inhibitory effect of light could be assessed for the polyphenol content (Scalbert *et al.*, 1988). Many different antioxidants have been included into culture media to prevent browning e.g. glutathione and ascorbic acid, but not all are effective. Some are only suitable for short term intervention, because they quickly become strong oxidising agents themselves e.g. ascorbic acid (Debergh and Read, 1991).

3. 4. Explant Selection

It is always important to ensure that explants are only taken from vigorous plants with no disease symptoms and no history of disease (George, 1993). To yield more hygienic explants, stock plants can be grown in greenhouses, it is important that the plants do not receive water by overhead irrigation but by devices that provide water directly in the pot or by capillarity. Overhead watering may stimulate the growth of surface micro-organisms and splashes from the compost may transfer organisms from soil onto leaves and stems. The age of the stock plant, the physiological age of the explant and its developmental stage as well as its size can determine the success of a procedure (Marks and Myers, 1994; Han *et al.*, 1997). When source plants have been grown under field conditions, it is sometimes difficult to obtain plants in a stable condition at any given time (Nomura *et al.*, 1998). The time of year that buds are collected for *in vitro* initiation can have a profound effect on culture establishment due to contamination levels and the physiological state of the buds (Skirvin *et al.*, 1986; Vieitiez *et al.*, 1986; San-Jose *et al.*, 1990; Silveira and Cottignies, 1994; Chalupa, 1996).

For most micropropagation work the explant of choice is an apical or axillary bud (Debergh and Read, 1991). Explant origin has been reported to affect culture growth *in vitro* (Norton and Norton, 1986; Welander, 1988; Webster and Jones, 1989; Marks and Myers, 1992). When apical or axillary buds are isolated at culture initiation, and plant growth regulators are applied *in vitro*, the direct interaction between them which would otherwise determine their particular growth habits on the stock plant is gone (Marks and Myers, 1992). It is expected that the exposure to exogenous plant growth regulators *in vitro* would overcome preconditioned responses carried over from the stockplant (Srivastava and Steinhauer, 1982) but this may not always be the case as demonstrated by Marks and Myers (1992) on *Betula pendula* and *Daphne ordora* cultures.

3. 5. Sterilisation of explant surfaces

Plants are invariably infested externally with fungi, bacteria, yeasts and animal pests (George, 1993). These organisms may be present on all external surfaces and are also likely to find their way into small crevices such as between bud scales and under ligules or stipules. For most kinds of tissue culture it is necessary to remove external microorganisms from plant tissues with chemical disinfectants, or sterilants before cultures are commenced (George, 1993). This is because bacteria and fungi are able to grow readily on plant culture media, which contain organic compounds such as sugars, amino acids and vitamins. The steps, which are necessary for effective decontamination, will depend on the nature of plant material and the kind of explant to be taken from it. In general, the part of the plant the explant to be dissected is first cleaned, any dead or superfluous tissue is removed and the material washed thorougly for several hours in water containing a wetting agent. This can help reduce the level of superficial contaminants and make the organisms more liable to be killed by subsequent treatments (George, 1993). Finally the chosen part of the plant is placed into a flask or screw top vial, disinfecting chemical treatments are applied and the container sealed.

There is no single antibiotic which will effectively control micro-organisms, and the use of antibiotics to rid cultures of bacterial contamination is problematic (Falkiner, 1988). It is far better is to eliminate the micro-organisms prior to culture (Parkinson *et al.*, 1996). A number of sterilisation treatments are available for plants, but some suffer from phytoxicity due in large part to the highly oxidising nature of the compounds (Abdul-Baki, 1974) and the high concentrations which must be used to produce effective levels of chlorine. Many woody species, such as *F. excelsior* and *Quercus robur,* are difficult to sterilise, and endemic contaminants are a common problem (Hammatt, 1994; Parkinson *et al.*, 1996). Mercuric chloride ($HgCl_2$) (0.1%) in combination with calcium hypochlorite ($Ca(OCl)_2$) (7%) is the usual sterilant for decontamination of *Q. robur* explants, the sterilisation protocol for which was optimised over a number of years to maximise sterilisation and minimise killing of the shoots by the sterilisation treatment (Pevalek-Kozlina, 1991). However, the toxicity of mercuric chloride presents difficulties for safe handling and disposal. Numerous studies on ash micropropagation have used mercuric chloride alone and in combination with calcium hypochlorite to sterilise the initial explants (Chalupa, 1990; Garelkova *et al.*, 1991; Silveira and Cottignies, 1994; Hammatt, 1997). Heavy metal ions would be toxic if introduced into a culture medium and so special care must be taken to thoroughly remove any traces by washing with sterile water. Calcium hypochlorite is commonly used to disinfest plant tissues and has been previously used on *F. excelsior* by Silveira and Cottignies (1994). The bactericidal action of hypochlorite solutions is due to both hypochlorous acid (HOCl) and the OCl^- ion. The former is probably much more active than the latter because the disinfecting efficiency of chlorine is best in slightly acid hypochlorite solutions (George, 1993). Pre-culture treatment of plants with elevated temperatures has been found to be effective for the inactivation and eventual removal of many viruses and some systemic bacterial diseases (Sivanesan and Waller, 1986). For this purpose it is

necessary to select a temperature regime which is above the optimum for growth of the micro-organisms but not lethal to the plant or plant material. Parts of plants, such as cuttings or bulbs and tubers, are often placed in hot water or hot air at 50-52°C for periods of 10 and 30 minutes (George, 1993).

3. 6. Media Composition

The successful establishment of a proliferating shoot culture will often depend not only on the selection of the most suitable explant, but also on the discovery of the correct combination of growth regulators, and/or the best salts medium for that organ or tissue. Medium formulations for culturing shoot tip and nodal explants have generally involved the use of Murashige and Skoog (1962) basal medium and explants can usually be established on the same medium formulation that can sustain shoot proliferation (Litz and Jaiswal, 1991). The results obtained for any one type of explant may vary between even closely related genotypes (Hammerschlag, 1982; San-Jose *et al.*, 1988, 1990). The basic nutrient requirements of cultured plant cells or organs are similar to those of whole plants. Plant tissue culture media are made up from some or all of the following components:

- Macronutrients: Provided from salts, which in the plant media dissociate into two ions. Calcium, magnesium, potassium, nitrogen, phosphorus and sulphur are all absorbed by the plant in the form of ions. The most important step in deriving a plant tissue culture medium is the selection of macronutrient ions in the correct concentration and balance (George, 1993).
- Micronutrients: The essential micronutrients for plant cell and tissue growth include iron, manganese, zinc, boron, copper, cobalt and molybdenum. Iron maybe the most critical of the micronutrients. Cobalt and iodine may also be added to certain media, but strict cell growth requirements for these elements have not been established yet. The essential micronutrient metals are components of plant cell proteins of metabolic and physiological importance. Micronutrients have roles in the functioning of genetic apparatus and several are involved with the activity of growth substances (George, 1993).
- Vitamins: The most frequently used vitamins in plant tissue culture media are thiamine (vitamin B_1), nicotinic acid (niacin) and pyridoxine (vitamin B_6) and *myo*-inositol.
- Carbohydrates: Only a limited number of plant cell lines have been isolated which are autotrophic when cultured *in vitro*. Research on *Cymbidium* (c. v. Lisa Rose No. 1), *Dianthus caryophyllus* L. and *Solanum tuberosum* L. (Kozai *et al.*, 1987; Kozai and Iwanami, 1988; Kozai *et al.*, 1988) has shown that chlorophyllous plantlets have photosynthetic ability *in vitro* and grew better in some cases under autotrophic rather than heterotrophic conditions. However, the costs of maintaining this type of production are high due to the elevated light levels required and associated the cooling costs. Normally for the culture of cells, tissues or organs, it is necessary to incorporate a carbon source into the medium. Sucrose is almost universally used for micropropagation purposes as it is so generally utilisable by tissue

cultures. The presence of sucrose in culture media specifically inhibits chlorophyll formation and photosynthesis, making autotrophic growth less feasible (George, 1993).

- Plant growth regulators: Some chemicals occurring naturally in plant tissues have a regulatory, rather than a nutritional role in growth and development. Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having an ability to modify plant growth by some other means, are usually termed plant growth regulators. The effects of growth regulators are generally not absolute and specific. The responses of cells, tissues and organs *in vitro* can vary according to cultural conditions, the type of explant and the plant genotype. Frequently a combination of two or more compounds of separate kinds is required, applied simultaneously or sequentially. Auxins and cytokinins are among the more common growth regulators used in plant culture. Auxins, such as IBA, are used to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis, especially in conjunction with cytokinins. They are capable of controlling processes such as cell growth and elongation, initiating cell division and are involved in the induction and development of meristems giving rise to either unorganised tissue, or defined organs. In organised tissue, auxins are responsible for the maintenance of apical dominance. Cytokinins, such as BAP and TDZ, added to the culture media can be used to overcome apical dominance and release lateral buds from dormancy (George, 1993).

Culture initiation is arguably the most critical stage of micropropagation, because the potential for multiple shoot generation lies within single buds (Marks and Myers, 1992). The problems imposed at this stage by micro-organism contamination, phenolics oxidation and tissue maturity, are well described in the literature, and many techniques have been developed to overcome them. The aim of the experimental work in this chapter was to optimise the initiation *in vitro,* of mature explants from a range of *F. excelsior* genotypes. This was achieved by testing a range of variables including explant selection (type of explant and harvesting time), sterilisation procedures and media composition (basal media, plant growth regulators and antioxidants).

3. 7. Materials and methods

A. Bud sources

Buds for experimentation were collected from the following three sources:

(1) Hedgerow plants (wild genotypes): In February 2002 and 2003 ash twigs were collected in north Co. Dublin, from hedgerows cut in the previous year. Dormant shoots were collected from 15-20 different coppiced hedgerow trees. Stem segments consisting of the dormant apical bud and 30 – 40cm of stem below were placed in plastic bags and stored.

(2) Grafted plants (selected genotypes): Superior ash trees were selected from the natural Irish population by Coillte (The Irish Forestry Board) on the basis of desirable traits such as clean

straight stems free from forking and disease. Each genotype is designated a unique identity number from 1 to 100. A similar collection of plus trees is maintained at Teagasc, Kinsealy. These trees are regularly pruned to promote a bushy form providing numerous shoots for experimentation (figure 3. 1). Similar stem segments containing dormant winter buds, as described for the hedgerow plants, were collected from the grafted plants in the stock plantation of selected 'plus' trees growing in the field under natural conditions at Kilmacurra, Co. Wicklow. The stem segments were stored at 4°C in the fridge until used in experiments. These trees were used as a source for dormant apical buds and for flushed summer shoots. Grafted plants of the selected plus trees, growing in 3 litre rose pots in the greenhouse were also used as a source of buds from naturally flushing shoots.

(3) Micropropagated plants (selected genotypes): Buds were taken in November 2001 from flushing shoots of plants, produced by micropropagation of selected plus trees, weaned to the glasshouse the previous summer. The plants were grown the greenhouse in large Rootrainers® (Ronaash Ltd., Kelso, Scotland).

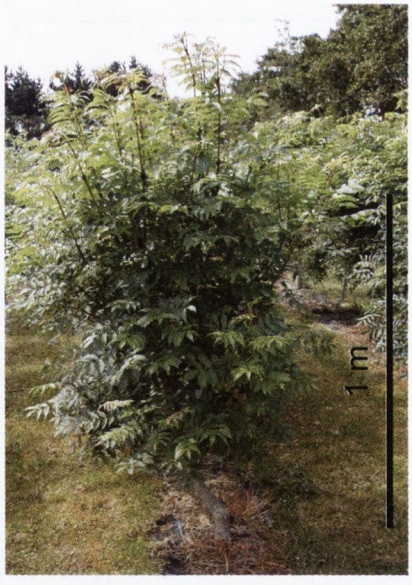


Figure 3. 1. A grafted plant of a selected plus tree (genotype 59) growing in the stock plantation at Kinsealy, Co. Dublin.

B. Culture media and maintenance

The standard medium used in the initiation experiments contained Lloyd and McCowns (1981) Woody Plant Medium (WPM) macronutrients and vitamins. The WPM micronutrients were at ten times normal concentration and Fe-EDTA was replaced by Sequestrene Fe 330 (see appendix 1), sucrose (20g/l) and agar (8.5g/l) were also added to the medium. Collectively, this media is

referred to as modified WPM, and was used as standard for the first two – six weeks of culture, depending on the explant type (section 3. 7. D). Explants were cultured on 6mls of modified WPM medium (unless stated otherwise) in 50mm Petri dishes (20mm deep) (Bibby Sterilin Ltd., Staffordshire, U. K.) sealed with Parafilm M® film (Pechiney Plastic packaging, USA). After this period, viable shoots were then placed into 200ml powder jars (Beatson Clark, Rotherham, U.K.) with screw top lids containing 30mls of modified WPM supplemented with 4.0mg/l BAP and 0.03mg/l IBA. Shoots were maintained on this media for four or five weeks before being transferred to fresh medium of the same composition. After the second culture period on the modified WPM with growth regulators the explants were transferred to a second WPM salts based medium containing modified macronutrients, micronutrients and vitamins (see appendix 1), sucrose (20g/l) and agar (8.5g/l). This media is hereby referred to as QRC media. After five weeks on the QRC media supplemented with 3g/l activated charcoal, the shoots were subcultured onto Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) modified by replacing MS vitamins with Gamborgs B5 vitamins (Gambourg, 1968) and containing Sequestrene Fe 330 instead of Fe-EDTA. This medium was supplemented with BAP (5mg/l), TDZ (0.55mg/l), IBA (2mg/l), sucrose (30g/l) and agar (9g/l), and collectively, is hereby referred to as M91 medium (see appendix 1). Explants were cycled every five to six weeks alternatively on the QRC medium with activated charcoal and M91 medium.

All media was adjusted to pH 5.8, by adding either hydrochloric acid (HCl) or sodium hydroxide (NaOH), prior to sterilisation in the autoclave at 121°C for 20 minutes at 15lb/in^2. Cultures were maintained in the growth room, at 22°C with 16-hour photoperiod, under Philips cool white fluorescent tubes (75 µmol m^{-2} s^{-1} at bench level).

C. Surface sterilisation

Standard sterilisation procedures were followed to decontaminate the explants prior to *in vitro* culture. These procedures were altered during experimentation as described in section 3. 7. F. 1.

Stems with apical and axillary buds were approximately 15mm in length for surface sterilization. The standard sterilisation procedure used for all explant material was to place the collected bud material into a 600ml beaker with a perforated foil lid to allow a stream of running water to wash over the buds for one hour (this step was not carried out as standard for dormant winter buds, section 3. 7. D. 1). This was followed by 15 minutes in 0.1% mercuric chloride, a five minute wash in sterile water, 15 minutes in 7% hypochlorite followed by three five minute washes in sterile water. For the dormant winter buds (section 3. 7. D. 1) as a standard treatment, the time in the sterilant solutions was increased to 40 minutes each. All sterilising procedures were carried out in 250ml closed vessels with approximately 10 explants per 250ml of solution. The closed vessels were placed on a mechanical shaker to agitate the solutions and ensure good contact between the

sterilant and the explants. The bleached ends of the stems were removed and explants placed in Petri dishes containing modified WPM medium (section 3. 7. B) (unless stated otherwise).

Contamination was defined as any explant showing fungal or bacterial contaminants growing over the explants as well as in or on the culture medium. All heavily contaminated buds with either fungi or bacteria were not viable for further culturing and were discarded. Some explants showing mild bacterial or fungal contamination were removed from the infected media and transferred to fresh clean media for further culturing, pads of filter paper soaked in a 2% (v/v) solution of Plant Preservative Mixture™ (PPM, Plant Cell Technology Inc., Washington DC, U. S. A.) (Plant Cell Technology, 1998) were placed around the explant to protect against reinfection (figure 3. 2).

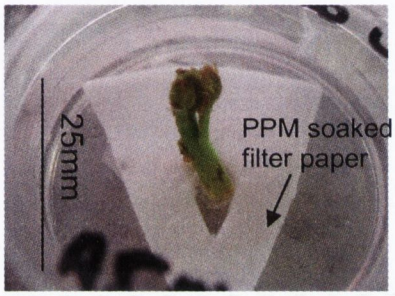
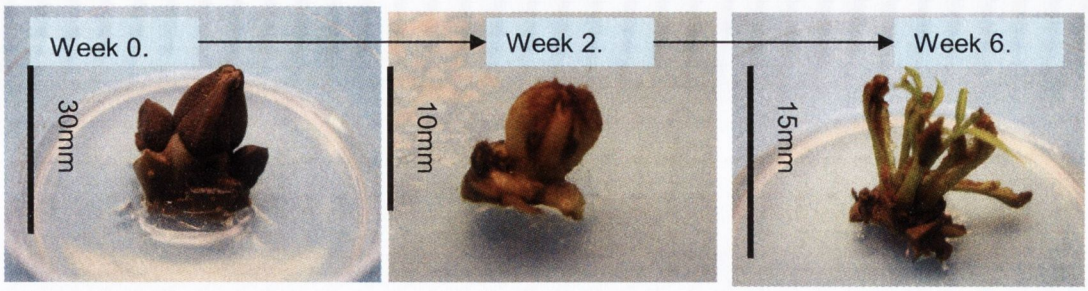


Figure 3. 2. An explant with a filter paper pad soaked in 2% Plant Preservative Mixture™ (PPM).

D. Physiological state of buds

Experiments were preformed using buds in three distinct physiological states.

(1) Dormant winter buds: Dormant apical buds were harvested from the hedgerow derived stem segments which were stored in the fridge (section 3. 7. A. 1) and from selected grafted plants from the field at Kinsealy (section 3. 7. A. 2). Explants were 30 – 40mm long bearing the apical bud, a pair of visible axillary buds and a portion of the stem below (figure 3. 3, stage 1). After sterilisation (section 3. 7. C.), the standard procedure was to culture the bud on modified WPM medium (refer to section 3. 7. B) in Petri dishes for two weeks, then the outer bud scales were removed from the apical bud and the shoot excised (figure 3. 3, stage 2), this was then placed on fresh modified WPM medium for four weeks. After this period, any viable shoots (figure 3. 3, stage 3) were placed into 200ml jars with screw top lids containing 30mls of modified WPM supplemented with 4.0mg/l BAP and 0.03mg/l IBA.



Stage 1. An apical explant on WPM medium.

Stage 2. The excised apical bud sprout.

Stage 3. A viable shoot.

Figure 3. 3. The three stages involved (post-sterilisation) in establishing a viable shoot culture from dormant winter buds.

(2) Naturally flushing shoots: Apical and nodal explants were harvested from the growing shoots of grafted plants from selected plus trees growing in the field and in the greenhouse at Kinsealy (section 3. 7. A. 2). Explants were also taken from the micropropagated selected trees (section 3. 7. A. 3). Apical explants were typically 10 – 20mm long and consisted of the apical bud and a pair of visible nodes beneath (figure 3. 4.). The nodal explants were of similar size and consisted of a single pair of opposite buds (figure 3. 4, pg 62). After sterilisation (section 3. 7. C.) the standard procedure was to culture the explants on modified WPM medium in Petri dishes for two weeks. After this period, any viable shoots were placed into 200ml jars with screw top lids containing 30mls of modified WPM supplemented with 4.0mg/l BAP and 0.03mg/l IBA. Shoots were maintained on this media for four or five weeks before being transferred to fresh medium. After the second culture period on the modified WPM with growth regulators they were transferred to QRC media (section 3. 7. B) supplemented with 3g/l activated charcoal. After five weeks on this media the shoots were subcultured onto M91 medium (section 3. 7. B). They were then cycled every five to six weeks alternatively on the QRC medium with activated charcoal and M91 medium.

(3) Forced winter buds: Stem cuttings prepared from the stem segments taken from grafted selected plus trees growing in the field at Kilmacurra (section 3. 7. A. 2), were forced into bud break via heat treatment to produce shoots for use as explants in experiments. The stem cuttings were approximately 100mm long and of two different types; one type was entire and had the apical bud present during heat treatment and forcing (figure 3. 5 a, pg 62). The second type had the apical bud removed immediately prior to heat treatment (figure 3. 5 b). The stem cuttings were forced into bud break by heat treating them in a 37^{o}C water bath, in direct contact with the water, for one hour. Immediately after heat treatment, the stem cuttings were planted at 5cm intervals, in seed trays containing a substrate of 3-4cm deep peat–vermiculite-perlite (v/v/v, 2:2:1). The basal 30mm portion of each stem cutting, including one pair of axillary buds, was immersed in the substrate.

Seed trays were then placed into a larger tray, which acted as a water reservoir so the bases of the cuttings were wet, (Silveira and Cottignies, 19946) (for a flow diagram of the steps involved in heat treating and forcing stem cuttings refer to figure 2. 3. in chapter 2). The stem cuttings were placed in the greenhouse for forcing under Philips SON 400W lights (16.5 μmol m^{-2} s^{-1} at bench level) with a 16-hour photoperiod, temperature in the greenhouse ranged from 15-22°C. In 2003 there was no heating provided in the greenhouse and temperatures ranged from 9-29 °C.

In the first type of cutting (figure 3. 5 a) the apical bud developed during forcing, in the second type of cutting either one or both of the axillary buds developed into a shoot (figure 5 b and c). In each case, the explant (figure 3. 5 d) for *in vitro* culture was an entire shoot derived from either the apical or the axillary buds of the stem cuttings (figure 3. 5 c). Shoots were collected from the forced stem cuttings and any leaf material removed. The standard sterilisation treatment (section 3. 7. C) was applied and the shoots cultured according to the standard procedure mentioned for explants from growing shoots (section 3. 7. D. 2).

E. Data collection and analysis

Each Petri dish contained one explant and there were 20-24 explants per treatment in each experiment described in section 3. 7. F. Experiments were arranged in a completely randomised design in the growth room. Recordings were made after four weeks (unless stated otherwise). Contamination was recorded in the *in vitro* cultures as any explant showing fungal or bacterial contaminants growing over the explants as well as in or on the culture medium. Viable buds were those, which were alive, green and in which some leaf development took place. The viable buds may have shown the presence of mild microbial infections that did not severely affect bud development.

Statistical analysis was performed using Statistica 6.0 statistical package. Percentage contamination and viability data was arc sin transformed for analysis by ANOVA. Mean separation was performed using Tukey's HSD test. Significance was recorded at $p \leq 0.05$.

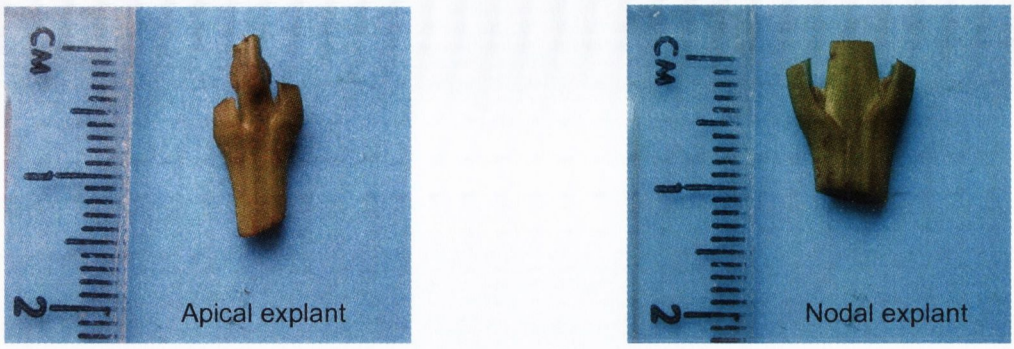


Figure 3. 4. A typical apical and nodal explant taken from naturally flushed shoots of selected 'plus' trees growing as grafted plants in the field or greenhouse, or from micropropagated plus trees growing in the greenhouse in Teagasc Kinsealy Research Centre, Co. Dublin.

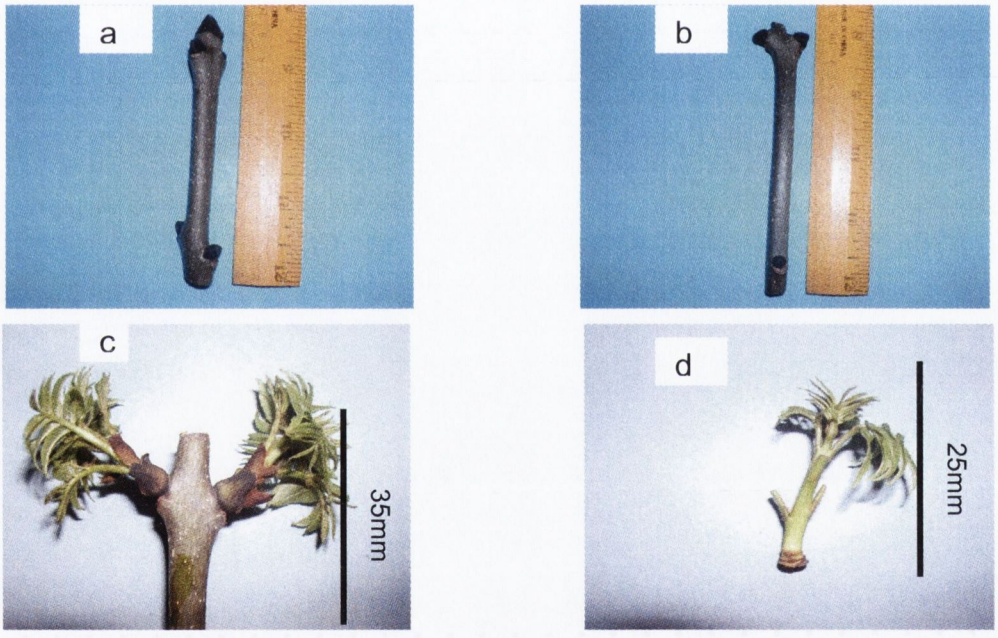


Figure 3. 5. The two types of stem cutting, with the apical bud (a) and without the apical bud (b), that were heat treated and forced in the greenhouse to produce shoots (c) used for initiating cultures of selected ash trees (d).

F. Experimental treatments

Experiments were performed using ash buds from different sources and in different physiological condition as discussed in section 3. 7. A and 3. 7. D.

(1) Experimental surface sterilisation treatments to improve the initiation of cultures.

Experiments to improve the standard sterilisation procedure (described in section 3. 7. C) were preformed using dormant winter buds (described in section 3. 7. D. 1) and naturally flushing shoots (described in section 3. 7. D. 2).

(1. 1) Dormant winter buds

The standard sterilisation procedure (section 3. 7. C) was altered for dormant winter buds by increasing the time in the sterilising solutions, mercuric chloride and calcium hypochlorite (table 3. 1. D1 and D2). Washing in running water was also investigated in the attempt to reduce explant contamination (table 3. 1. D3). Pre-sterilisation treatments were included which involved immersion in a water bath at 38°C for 12 hours, 38 °C for 1 hour or 45°C for 1 hour (table 3. 1. D4, D5 and D6). Controls received no heat treatment and were sterilised using method D3 outlined in table 3. 1. After the sterilisation treatments all buds were cultured according to the method described in section 3. 7. D. 1. The development of contamination and explant viability was recorded over a four-week period.

(1. 2.) Shoots that had flushed naturally in situ

The standard sterilisation procedure (section 3. 7. C) was used as a control for this set of experiments. Flushed shoots were collected in the field at Kinsealy (described in section 3. 7. D. 2) and were used to test the effects of three sterilisation pre-treatments and a post sterilisation dip on contamination frequency. After sterilisation treatments all buds were cultured according to the method described in section 3. 7. D. 2. The treatments were:

1. The control – standard sterilisation procedure (described in section 3. 7. C).
2. One minute in 70% ethanol prior to the mercuric chloride sterilisation step.
3. A 10 minute wash in warm water and teepol® (two drops per litre) instead of one hour washing in running water.
4. 10 minute wash in warm water (±25°C) and teepol® followed by a two hour wash in running water.
5. A 10 second dip in copper sulphate ($CuSO_4$) solution (100mg/l) post sterilisation.

At the end of the initiation experiments a comparison was made between the contamination levels in experiments involving explants taken from greenhouse grown grafted plants of selected genotypes and field grown grafted plants of selected genotypes, which were given the same standard sterilisation procedure prior to *in vitro* culture.

Table 3. 1. Sterilisation treatments investigated for dormant winter buds.

Code	*Running water (minutes)*	*Water bath*	*Mercuric chloride (0.1%) (minutes)*	*Calcium hypochlorite (7%) (minutes)*
D1	0	-	40	40
D2	0	-	60	60
D3	60	-	60	60
D4	-	12 hrs at 38°C	40	40
D5	-	1 hr at 38°C	40	40
D6	-	1 hr at 45°C	40	40

(2) Initiation of shoot cultures in vitro using heat treated dormant winter buds.

All winter buds were heat treated prior to experimentation; heat treatment of buds was administered in water. Two methods of bud treatment (described below) were used to investigate the initiation of cultures using dormant winter buds:

(2. 1) Winter buds (section D. 1) (heat treated) ⟶ *sterilisation* ⟶ in vitro *culture.*

Attempts were made to establish viable cultures using dormant apical buds (described in section 3. 7. D. 1) using heat treatments to reduce contamination (described in section 3. 7. F 1. 1) and break bud dormancy. Apical explants were immersed in a water bath then sterilised by method D4 outlined in table 3. 1. All buds were cultured according to the method described in section 3. 7. D. 1.

(2. 1. i) To test the effect of heat treatment on the viability of buds from different genotypes of *F. excelsior,* explants were taken from selected trees of genotype identity 5, 7 and 49 (section 3. 7. A. 2) and wild material (section 3. 7. A. 1). The buds were harvested in mid February 2002 and 2003 and given heat treatment at 38°C for 12 hours.

(2. 1. ii) To test the effect on bud viability of administering heat treatment during different stages in dormancy, apical explants of ash genotype 7 (section 3. 7. A. 1) were harvested in January, February and March 2002 and treated at 38°C for 12 hours.

(2. 1. iii)To test the effect of a higher temperature (45°C) over a shorter time period (one hour), apical buds of selected genotype 5 were harvested and treated in February 2002 and February 2003. In addition, this treatment was tested on wild material harvested in February 2003. After heat treatment and sterilisation (according to method D6, table 3. 1.) the explants were divided into two groups and placed in the growth room in either light or dark conditions to test whether culturing in

the light or dark after heat treatment had an effect on bud viability. Controls received no heat treatment, were sterilised (according to method D3, table 3. 1.) and were in the light.

(2. 1. iv) To test the effect of the natural antioxidant glutathione on the viability of heat treated buds, the apical buds were heat treated at 38°C for 12 hours, then placed on modified WPM medium containing 0mg/l, 0.01mg/l, 0.1mg/l, 1 mg/l or 5mg/l reduced glutathione (GSH) in 2002. This experiment was repeated in 2003; with the heat treated explants placed on modified WPM medium containing 0mg/l, 1mg/l, 5mg/l, 10mg/l or 30mg/l reduced glutathione. For this experiment, the standard culturing procedure described for winter buds in section 3. 7. D. 1 was adjusted. Buds were cultured for four weeks on the experimental media without transfer or dissection, after this period any viable shoots were then placed into 200ml jars with screw top lids containing 30mls of modified WPM supplemented with 4.0mg/l BAP and 0.03mg/l IBA.

(2. 2.) Winter buds (heat treated) → *forced* → *sterilisation* → in vitro *culture.*

In these experiments, stem cuttings (section 3. 7. D. 3) were heat treated at 37°C for one-hour in the water bath then grown under forcing conditions in the green house (described in section 3. 7. D. 3). Dormant stem cuttings, from 14 selected genotypes in March 2002 and from wild genotypes in April 2003, were heat treated then forced in the greenhouse. The viability *in vitro*, of shoots derived from two different types of stem cutting, those with the apical bud and those without the apical bud (figure 3. 5 a and b), was tested in 2002. In 2003, the viability *in vitro* of apical bud derived shoots taken at two different stages of bud development; three weeks after heat treatment and six weeks after heat treatment, was tested. The treatment of the stem cuttings and shoots for *in vitro* culture is described in section 3. 7. D. 3.

(3) Initiation of naturally flushing shoots in vitro.

All the explants used in these experiments were collected from naturally flushing shoots of grafted plants of selected genotypes growing in field conditions in summer at Kinsealy, Co. Dublin (section 3. 7. D. 2). The standard sterilisation procedure (described in section 3. 7. C.) was used, after sterilisation all buds were cultured according to the standard procedure (unless stated otherwise) described in section 3. 7. D. 2.

(3. 1. i) To investigate the viability *in vitro* of apical explants compared to nodal explants of summer shoots. The shoot apices and nodes of selected genotypes 5, 7 and 49 were collected in June 2002. The apical explants consisted of the terminal bud and two axillary buds and the nodal explants consisted of a single node (figure 3. 4). Explants were approximately 10mm long.

(3. 1. ii) To investigate the effect of time of culture initiation on viability and contamination *in vitro*, nodal explants of selected genotype 5, 7 and 49 were collected in June, July and August 2002 and May, June and July 2003.

(3. 1. iii)To investigate the effect of initiation medium on explant viability *in vitro*, nodal explants of selected genotype 57, 58, 3 and 4 were harvested in June 2002. After sterilisation (section 3. 7. C) the explants were placed on modified WPM medium or M91 medium (described in section 3. 7. B of this chapter) in Petri dishes. After two weeks, explants were transferred to the same type of media in 200ml glass jars. One month later, any surviving explants were placed on M91 media in 200ml glass jars.

(3. 1. iv) Nodal explants of selected genotypes 5, 7 and 49 were used to investigate the effects on explant viability, of adding the supplements adenine sulphate (15 mg/l) and ascorbic acid (10 mg/l) to the modified WPM medium. Explants from each genotype were cultured on both modified WPM medium and modified WPM medium containing the supplements adenine sulphate and ascorbic acid. Cultures were maintained on this media in Petri dishes for two weeks, then transferred to fresh media in 200ml glass jars for a further two weeks. Any surviving explants were transferred to M91 medium for one months culturing.

(3. 2.) To investigate the use of micropropagated plants (section 3. 7. A. 3) as a source of viable buds, apical and nodal explants were taken from the growing shoots of selected genotype 47, 49 and 72 and from an unselected genotype F5, in November 2001. Explants were sterilised according to the standard procedure (section 3. 7. C) and cultured on 6mls of M91 medium with or without 1.1mg/l TDZ (instead of the standard 0.55mg/l TDZ) in Petri dishes, for one month before being transferred to 30mls of QRC media supplemented with 3g/l activated charcoal in jars.

3. 8. Results

(1) Sterilisation treatments to reduce contamination.

Experiments on sterilisation methods were performed using:

(1. 1) Dormant winter buds (apical explants).

(1. 2) Shoots that had flushed naturally *in situ.*

(1. 1) Dormant winter buds.

The standard sterilisation procedure outlined in section 3. 7. C., was altered for dormant winter buds by increasing the time in the sterilising solutions; mercuric chloride and calcium hypochlorite (table 3. 1. D1 and D2). Washing in running water was also investigated in the attempt to reduce explant contamination (table 3. 1. D3). Pre-sterilisation treatments were included which involved immersion in a water bath at 38°C for 12 hours, 38 °C for 1 hour or 45°C for 1 hour (table 3. 1. D4, D5 and D6). Controls received no heat treatment and were sterilised using method D3 outlined in table 3. 1.

The results are shown in table 3. 2. All buds given no heat treatment or treated at 38°C for one hour showed 100% contamination. A sterilisation pre-treatment by heat of either 12 hours at 38°C (D4) or 1 hour at 45°C (D6) significantly reduced contamination in each case to 40% (Tukey's HSD $p<0.001$) (table 3. 2), whereas one hour at 38°C was not effective. The viability of heat treated buds at 38°C for 12 hours was 45% and was 47.5% for buds treated at 45°C for 1 hour, this was not a significant difference between the two treatments (Tukey's HSD, $p=0.96$). Bud viability in all other treatments was significantly lower (0% for all four treatments), due to high levels of contamination (Tukey's HSD, $p<0.0001$). After heat treatment some uncontaminated buds turned brown and died, the remaining viable buds lived for a further two to three months but failed to proliferate and eventually died.

(1. 2) Shoots that had flushed naturally in situ

Material collected in the field from the selected genotypes was used to test the effects of three sterilisation pre-treatments and one post-sterilisation treatment on contamination levels (section 3. 7. F. (1. 2.)). Shoots sterilised by the standard procedure showed 100% contamination, all the pre-treatments tested reduced contamination compared to the controls (see table 3. 3) A post sterilisation dip treatment for 10 seconds in 100mg/l copper sulphate solution after sterilisation, reduced contamination significantly compared to the control (Tukey HSD, $p<0.02$) (table 3. 3). However, a sterilisation pre-treatment of 10 minutes washing in warm water and teepol® followed by two hours washing in running water reduced contamination significantly compared to all other treatments (Tukey's HSD, $p<0.02$).

When the contamination levels from experiments involving explants taken from greenhouse grown grafted plants or field grown grafted plants of selected genotype were compared, explants taken from the greenhouse grown grafted plants and given the standard sterilisation treatment showed significantly less contamination (27.1%) compared to those taken from field grown grafted plants of selected genotype (95.7%) (Chi square test, $p<0.00001$).

(2) Initiation of shoot cultures in vitro *using heat treated dormant winter buds.*

All winter buds were heat treated prior to sterilisation and culture initiation; two methods were applied; heat treatment alone (section 3. 7. F. (2. 1)) and heat treatment followed by forcing (section 3. 7. F. (2. 2)).

(2. 1) Winter buds (heat treated) ⟶ *sterilisation* ⟶ in vitro *culture.*

Attempts were made to establish viable cultures from dormant apical buds by using several heat treatments to reduce contamination (described in section 3. 7. F. (1. 1)) and break bud dormancy. Apical explants were heat treated, then sterilised by method D4 outlined in table 3. 1., and cultured (section 3. 7. D. (1)).

Table 3. 2. The effect of sterilisation treatments on contamination levels in cultured dormant apical buds.

Code	*Running water (mins)*	*Water bath*	*Mercuric chloride (0.1%) (minutes)*	*Calcium hypochlorite (7%) (minutes)*	*Contamination (%)*
D1	0	-	40	40	100[a*]
D2	0	-	60	60	100[a]
D3	60	-	60	60	100[a]
D4	0	12 hrs at 38°C	40	40	40[b]
D5	0	1 hr at 38°C	40	40	100[a]
D6	0	1 hr at 45°C	40	40	40[b]

*Different letters within columns indicate a significant difference according to Tukey's HSD ($p<0.0002$).

Table 3. 3. The effect of pre-sterilisation treatments on contamination in flushed summer shoots.

No.	*Pre-Treatment**	*Contamination (%)*
1.	None (control)	100 [a**]
2.	One minute in 70% Ethanol	85.7 [ab]
3.	$CuSO_4$ dip (post-sterilisation)	60 [b]
4.	10 minutes in warm water (±25°C) and teepol®	80.9 [ab]
5.	10 minutes teepol® wash + 2hr wash in running water	22.7 [c]

* All sterilisation pre-treatments were followed by the standard sterilisation procedure (see materials and methods section C). ** Different letters within columns indicate a significant difference ($p<0.05$) according to Tukey's HSD.

(2. 1. i) The effects of a heat treatment on contamination and viability of buds was recorded for individual selected genotypes 5, 7 and 49 as well as a collection of wild genotypes over two years (section 3. 7. F. (2.1.i)). The results are summarised in table 3. 4. In 2002 the contamination level was lowest for all material tested compared to 2003. In 2003 contamination rates were very high in all the selected genotypes but was significantly lower for the collection of wild genotypes (Tukey's HSD, $p<0.0003$). There were significant differences in the viability of buds of different genotype treated in 2002 (table 3. 4.). Genotype A5 had significantly more viable buds two months after treatment than any other genotype tested (Tukey's HSD, $p<0.0002$). In 2003 viability was very low for all the genotypes tested, this was due to the very high contamination rates.

Table 3. 4. The effect of heat treatment (38°C for 12 hours) on viability and contamination of dormant apical buds of different genotype.

Genotype	*Contamination (%)*		*Viability (%)*	
	2002	*2003*	*2002*	*2003*
5	10^{a*}	95^{a}	65^{a}	5^{a}
7	0^{a}	100^{a}	10^{b}	0^{a}
49	0^{a}	100^{a}	0^{b}	0^{a}
Wild	50^{b}	55^{b}	5^{b}	10^{a}

*Different letters within columns indicate a significant difference according to Tukey's HSD ($p<0.0003$).

(2. 1. ii) The effectiveness of applying a heat treatment to dormant buds at three different periods in the season was evaluated in 2002 using buds from the selected genotype 7 (section 3. 7. F. (2.1.ii)) and the results are summarised in figure 3. 6. The contamination levels recorded were 0%, 6.67% and 75% for the months of January, February and March respectively, contamination in March was mostly from bacteria. There were significant differences in viability between the three months tested (Chi square test, $p<0.00001$). Buds treated in January showed 100% viability one month after treatment, this was significantly higher than viability levels in both February (40%) and March (10%) (Tukey's HSD, $p<0.0002$) (figure 3. 6.). Viable buds lived a further two to three months but failed to proliferate and eventually died.

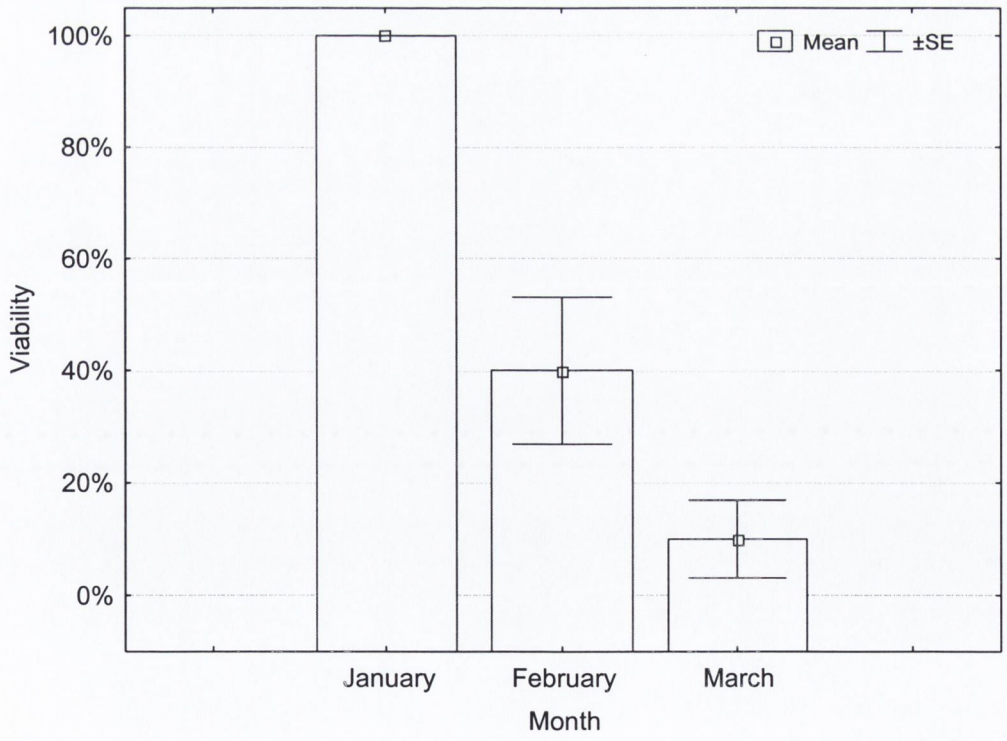


Figure 3. 6. The effect of heat treatment (38°C for 12 hours) on viability *in vitro* of dormant apical buds in January, February and March 2002 for selected genotype 7.

(2. 1. iii) The effects of applying a higher temperature of 45°C for a shorter duration of one hour was tested on apical buds from selected genotype 5 during February 2002 and 2003, as well as with buds from wild genotypes in February 2003 (section 3. 7. F. (2.1.iii)) Furthermore after heat treatment and sterilisation the explants were divided into two groups and placed in the growth room in either light or dark conditions to test whether culturing in the light or dark after heat treatment had an effect on bud viability. Controls received no heat treatment, were sterilised and were cultured in the light. Table 3. 5. shows the results obtained for selected genotype 5 in 2002 and 2003, and wild genotypes in 2003. Without a heat treatment, 89-100% of buds were contaminated, therefore the control buds had consistently low viability rates over two years, 2002 and 2003 for all genotypes tested. Heat treatments significantly improved the percentage of sterile buds obtained in each year. Furthermore, culturing buds in the dark did not affect the contamination rate recorded in each year. In both years, a heat treatment improved bud viability when buds were cultured in the light (2002: 31.6% viability, 2003: 57.1% viability, NS: 55% viability) (table 3. 5.). Culturing heat treated buds in darkness reduced bud viability consistently over two years, 2002 and 2003 for all genotypes tested.

Table 3. 5. The effect of heat treatment (45°C for 1 hour) followed by culture in the light or dark on viability and contamination of dormant apical buds from selected genotype 5 in 2002 and 2003 and wild genotypes in 2003.

Treatment	*Contamination (%)*			*Viability (%)*		
	2002	2003	Wild	2002	2003	Wild
Control (no heat, light)	100 [a*]	89.5 [a]	95 [a]	0 [a]	10.5 [a]	5 [a]
45°C for 1 hour (light)	21 [b]	14.3 [b]	30 [b]	31.6 [b]	57.1 [b]	55 [b]
45°C for 1 hour (dark)	15 [b]	30 [b]	30 [b]	15 [a b]	0 [a]	45 [b]

*Different letters within columns indicate a significant difference according to Tukey's HSD (2002: $p<0.02$, 2003: $p<0.0005$, NS: $p<0.01$).

(2. 1. iv) Buds cultured from dormant winter shoots frequently showed brown staining of the agar due to oxidation induced browning and the production of phenols from the explants. The effect of the natural antioxidant glutathione during culture on the viability of heat treated buds (38°C for 12 hours) was evaluated. Reduced glutathione was tested at 0mg/l, 0.01mg/l, 0.1mg/l, 1 mg/l or 5mg/l in 2002. This experiment was repeated in 2003, at concentrations of 0mg/l, 1mg/l, 5mg/l, 10mg/l or 30mg/l reduced glutathione. Buds were heat treated, sterilised and cultured as described in the section 3. 7. F. (2.1.iv). The contamination levels were 29% in 2002, and 59% in 2003. In 2002 buds cultured on 5.0mg/l glutathione were significantly more viable after one month compared to controls cultured on 0mg/l glutathione (Tukey's HSD, $p<0.03$) (table 3. 6.). When the experiment was repeated in 2003 the results proved inconclusive due to high contamination levels in each treatment, however it was observed that there were less explant deaths due to browning on the media with 1mg/l glutathione compared to the other media tested.

Table 3. 6. The effect of culturing heat treated (38°C for 12 hours) dormant apical buds on WPM medium containing five concentrations of glutathione (0 – 5 mg/l).

Glutathione (mg/l)	*Viability (%)*
0	15^{a*}
0.01	30^{ab}
0.1	25^{ab}
1	10^{a}
5	55^{b}

*Different letters within columns indicate a significant difference according to Tukey's HSD ($p<0.03$).

(2. 2) Winter buds (heat treated) ⟶ *forced* ⟶ *sterilisation* ⟶ in vitro *culture.*

In these experiments stem cuttings were heat treated at 37°C for one-hour in the water bath then grown under forcing conditions in the green house (section 3. 7. F. (2. 2.)). The shoot explants for culturing were derived either from the apical bud or axillary buds of heat treated and forced stem cuttings. Stem cuttings from 14 selected genotypes were used in March 2002. In 2002 the contamination rate was 47.8% over all the explant types, and the apical bud derived shoots (66.7% viability) were significantly more viable *in vitro* than the axillary bud derived shoots (27.1% viability) (Chi square test, $p<0.0002$). It was noted that during this experiment the longer, more developed apical bud shoots were surviving better *in vitro* than the shorter ones. In March 2003, the contamination level was 55.8% for all the explant types. The apical bud derived shoots were divided into two categories; apical shoots (S) which were collected three weeks after the heat treatment and were less than 10mm long, and apical shoots (L) which were collected six weeks after heat treatment and were greater than 10mm in length. The longer, more developed apical bud shoots (L) were significantly more viable *in vitro* compared to the short, younger shoots (S) (Chi square test, $p<0.0008$).

Although explants may have remained viable for some months by subculturing of material from the experiments described in this section, no proliferating shoot cultures were established from any material initiated *in vitro* from dormant winter buds.

(3) Initiation of naturally flushing summer shoots in vitro.

All the explants used in these experiments were collected from naturally flushing shoots of grafted plants of selected genotypes growing in field conditions in Kinsealy, Co. Dublin. The explants were sterilised and cultured as described in section 3. 7. F. (3).

(3. 1. i) The effect of explant type was evaluated by culturing apical and nodal explants of selected genotypes 5, 7 and 49 (section 3. 7. F. (3.1.i)). The contamination levels in this experiment were over 90% for all genotypes, bacteria were the main pathogens affecting the explants. Only the

heavily infected explants were discarded, the remainder were treated with the filter paper pads soaked in 2% PPM as described in the materials and methods section C. The results for each genotype are shown in table 3. 7. Overall, the viability of nodal explants (40.8%) was significantly higher than apical bud explants (2.4%) (Chi square test, $p<0.0001$). In addition, there was no difference among the three genotypes for viability of apical buds whereas there was a significant difference among the viability of nodal explants (One-way ANOVA, $p<0.0001$). Further analysis of the combined viability values for apical and nodal explants revealed a significant interaction between genotype and explant type (Factorial ANOVA, $p<0.0005$, figure 3. 7).

Table 3. 7. The effect of explant type on viability of flushed summer shoots of different genotype.

Genotype	*Explant*	
	Apical	*Nodal*
5	$7.7\%^{aA*}$	$41.2\%^{aA}$
7	$0\%^{aA}$	$75.0\%^{bB}$
49	$0\%^{aA}$	$6.0\%^{cA}$
Total	$2.4\%^{A}$	$40.8\%^{B}$

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.007$) according to Tukey's HSD.

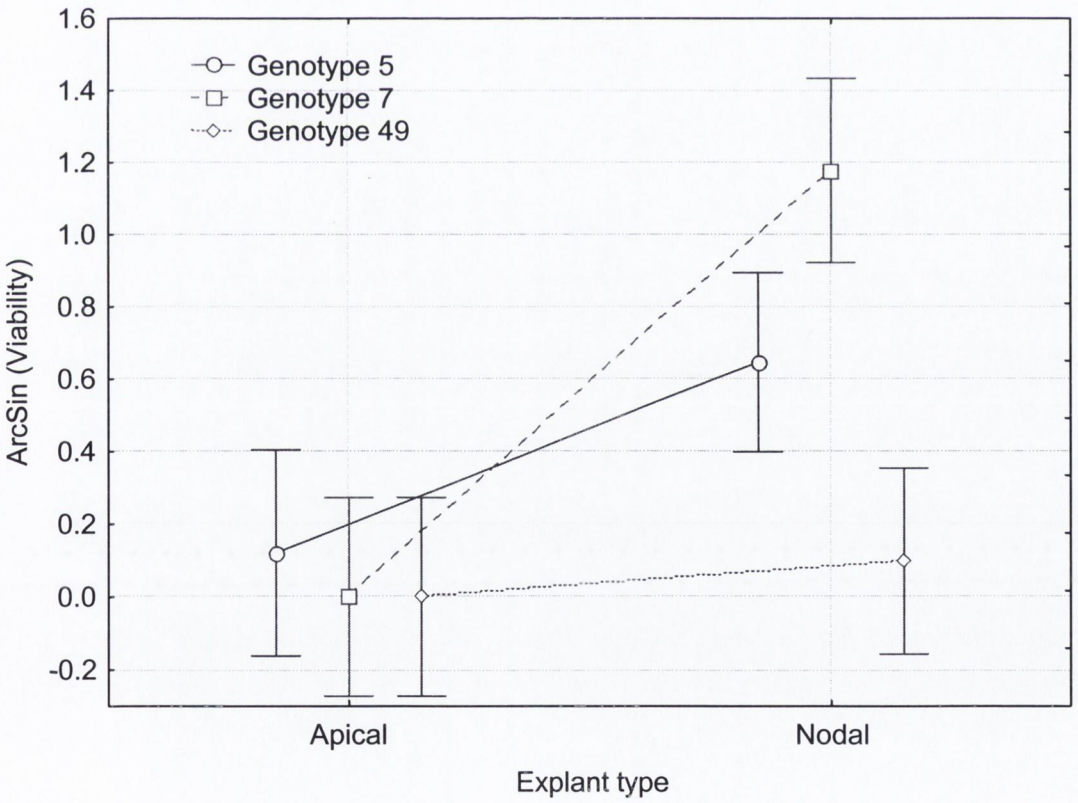


Figure 3. 7. The interaction between genotype (5, 7 and 49), explant type (apical and nodal) and viability (vertical bars denote 95% confidence interval).

(3. 1. ii) Nodal explants were used subsequently in experiments to study the effects of the dates of shoot collection; June, July and August in 2002 and May, June and July 2003, on the contamination level and viability of cultured nodes. The selected genotypes 5, 7 and 49 were used. The contamination rates were high for each month in 2002 from the combined data of all three genotypes (table 3. 8). There was a significant difference in percentage viability of explants initiated during the different months (Chi square, $p<0.000001$; table 3. 8). Explants initiated in July were significantly more viable *in vitro* compared to those initiated in either June or August (Tukey's HSD, $p<0.00003$; table 3. 8). The viability of buds cultured in these three periods is summarised in table 3. 9. It shows the highest viability for buds cultured in July. In addition, there was no significant difference in percentage viability, over the combined three month period, between the genotypes (5: 17.2%, 7: 13.5% and 49: 6.7%) (Chi square test, $p=0.2087$). There was a significant interaction between genotype, month initiated and viability of the explants in 2002 (Factorial ANOVA, $p<0.02$; table 3. 9).

The same genotypes were again tested for contamination levels and viability of buds when cultured in May, June and July of 2003. Contamination rates increased from 55.4% in buds cultured in May to 96.7% for those cultured in July (Chi square test, $p<0.000001$) and overall was significantly higher for genotype 5 (96.8%) compared to either of the other two, genotype 7 (73.4%) or 49 (73.4%). Furthermore there was a significant interaction for contamination levels between genotype and the month of culture initiation (Factorial ANOVA, $p<0.000001$; table 3. 11). Regarding culture viability in 2003, there was no significant difference in percentage viability of explants initiated in May, June or July (Chi square test, $p=0.141$; table 3. 10). However, there was a significant difference in percentage viability, over the three month period, between the three genotypes A5 (6.3%), A7 (39.1%) and JK49 (6.1%) (Chi square test, $p<0.00001$). There was no significant interaction between genotype, month of initiation and viability of the explants (Factorial ANOVA, $p=0.6561$). The overall contamination level and viability of buds from flushed shoots in each of the two years was compared. Contamination levels decreased significantly from 97.2% in 2002 to 81.2% in 2003 (Chi square test, $p<0.0000001$). There was a significant difference in viability between the two years (2002: 12.4%, 2003: 17.4%) (Chi square test, $p=0.199$). However, a significant interaction was identified between genotype, year and viability of the explants whereby viability levels in genotypes 5 and 49 decreased from 2002 to 2003 but the viability level in genotype 7 increases between the two years tested (Factorial ANOVA, $p<0.0002$) (table 3. 12).

Table 3. 8. The effect of initiation time on the viability and contamination rate of flushed summer shoots initiated in June, July and August 2002 (combined results for genotypes 5, 7 and 49).

Month of culture	*Contamination (%)*	*Viability (%)*	*Temperature (°C)*
June	94.4[a]	4.2[a*]	13.4**
July	97.8[a]	42.2[b]	14.8
August	100[a]	0[a]	15.8

* Different letters within columns indicate a significant difference ($p<0.00003$) according to Tukey's HSD. ** Average monthly temperature, data recorded at Teagasc Kinsealy Research Centre, Co. Dublin.

Table 3. 9. The effect of time of initiation and genotype on the viability of flushed summer shoots *in vitro* in 2002.

Genotype	*Month of culture*		
	June	*July*	*August*
5	4.3%[aA*]	60%[aB]	0%[aA]
7	4.2%[aA]	46.6%[aB]	0%[aA]
49	4.2%[aA]	20%[bA]	0%[aA]

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.003$) according to Tukey's HSD.

Table 3. 10. The effect of time of initiation on viability and contamination rates of flushed summer shoots in 2003 (combined results for genotypes 5, 7 and 49).

Month of culture	*Contamination (%)*	*Viability (%)*	*Temperature (°C)*
May	55.4[a]	24.6[a*]	10.9**
June	92.3[b]	12.3[a]	13.9
July	96.7[b]	14.5[a]	16.2

*Different letters within columns indicate a significant difference ($p<0.00003$) according to Tukey's HSD. ** Average monthly temperature, data taken from Met Eireann (www.met.ie).

Table 3. 11. The effect of initiation time and genotype on contamination rates in flushing summer shoots in 2003.

Genotype	*Month of culture*		
	May	*June*	*July*
5	95.2%[aA*]	95.2%[aA]	100%[aA]
7	47.6%[bA]	82.6%[aB]	90%[aB]
49	26%[bA]	100%[aB]	100%[aB]

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.00003$) according to Tukey's HSD.

Table 3. 12. The effect of initiation year on the viability of flushed summer shoots of different genotype initiated in 2002 and 2003.

Genotype	*Year*	
	2002	*2003*
5	17.2%^{aA*}	6.3%^{aA}
7	13.5%^{aA}	39%^{bB}
49	6.6%^{aA}	6.1%^{aA}

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.007$) according to Tukey's HSD.

(3. 1. iii) To investigate the effect of initiation medium on explant viability *in vitro*, nodal explants of genotype 57, 58, 3 and 4 were harvested in June 2002 and cultured on modified WPM medium and M91 medium (for media constituents see section 3. 7. B). Explant viability for all four genotypes combined was significantly higher modified WPM media (30.7% viability) compared to M91 media (10 % viability) (Chi square test, $p=0.03$). There was no significant interaction between genotype and media (Factorial ANOVA, $p<0.78$; table 3. 13)

Table 3. 13. The effect of initiation media on the viability of flushed summer shoots of different genotype initiated in 2002.

Genotype	*Media*	
	Modified WPM	*M91*
57	44.4% *	10.0%
58	33.3%	16.7%
3	11.1%	0%
4	33.3%	25.0%

*There were no significant differences at the 5% level.

(3. 1. iv) In efforts to improve bud viability, modified WPM medium was supplemented with the organic nutrient adenine sulphate (15mg/l) and the antioxidant ascorbic acid (10mg/l). The supplemented medium was compared to unsupplemented medium for the three selected genotypes 5, 7 and 49 using nodal explants as described in section 3. 7. D. (2). The results are shown in table 3. 14. Supplementing the culture medium significantly reduced bud viability *in vitro* from 33.3% to 13.3% for all genotypes tested (Chi square test, $p=0.02$). In comparing the interaction between supplemented and unsupplemented media and the three genotypes used, no significant interaction was found (Factorial ANOVA, $p=0.5249$; table 3. 14).

Table 3. 14. The effect of modified WPM media supplemented with adenine sulphate (15mg/l) and ascorbic acid (10mg/l) compared to unsupplemented media on the viability of flushed summer shoots of different genotype initiated in 2002.

Genotype	*Media*	
	Supplemented	*Unsupplemented*
5	13.3% *	40%
7	26.7%	53.3%
49	0%	6.67%

* There were no significant differences at the 5% level.

(3. 2) To investigate the use of micropropagated plants (section 3. 7. A. (3)) as a source of viable buds apical and nodal explants were taken from the growing shoots of selected genotype 47, 49 and 72 and from an unselected genotype F5, in November 2001. The results are shown in table 3. 15. There was a significant difference in the amount of contamination recorded *in vitro* between genotypes (Chi square test, $p<0.00001$). Genotype F5 had the highest contamination level (66%) compared to the other genotypes tested (table 3. 15). Viability levels were similar between genotypes over both media tested (M91 medium with or without 1.1mg/l TDZ) (genotype F5: 70%, 47: 86.4%, 49: 66.7% and 72: 76.5% viability) (chi square test, $p=0.39$) and between both media overall the genotypes tested (media with TDZ: 76.7%, media without TDZ: 71.6% viability) (Chi square test, $p=0.48$). There was no interaction between genotypes and the presence or absence of TDZ in the initiation media for bud viability (Factorial ANOVA, $p=0.27$).

Table 3. 15. Contamination and viability rates for three selected genotypes and one wild genotype initiated from micropropagated mother plants on media with or without 1.1mg/l TDZ.

Genotype	*Contamination (%)*	*Viability (%)*	
		With TDZ	*Without TDZ*
F5	66^{a*}	70.8^{a}	69.2^{a}
47	13.6^{b}	83.3^{a}	90.0^{a}
49	37.5^{ab}	58.3^{a}	75.0^{a}
72	31.4^{b}	88.0^{a}	65.4^{a}

* Different letters within columns indicate a significant difference ($p<0.001$) according to Tukey's unequal N HSD.

Explants taken from the micropropagated mother plants became truly established *in vitro* and produced proliferating cultures after a few subcultures. Explants from naturally flushing summer shoots were more successful at surviving *in vitro* than those taken from dormant winter buds. Although some explants may have survived for up to 12 months after initiation most failed to produce proliferating cultures.

3. 9. Discussion

The aim of the initiations section of this thesis was to identify the optimum method to establish clean, healthy shoot cultures of selected mature Irish ash trees.

The results in section 3. 8. (1) highlight the difficulties in establishing uncontaminated shoot cultures of ash *in vitro*. The most problematic pathogens affecting the explants in both the dormant and flushed shoots were bacteria, although fungal contamination was also present. For both the dormant winter buds and flushed summer shoots a pre-sterilisation treatment was necessary to decontaminate the explants. Contamination levels were reduced in winter buds when given either heat treatment of long duration at 38°C or of short duration at 45°C prior to sterilisation. For the flushed summer shoots, the most successful pre-sterilisation treatment for decontaminating explants was a wash in warm water (±25°C) containing the wetting agent teepol® followed by washing in running water for two hours. A post-sterilisation dip in $CuSO_4$ was also shown to be effective in reducing contamination in flushed summer shoots. The dormant winter buds proved more difficult to decontaminate than the flushed shoots and needed more than twice as long in the sterilising solutions.

Hammatt (1997) noted that attempts to introduce new ash material into culture were hampered by difficulties in surface sterilisation, and that testing combinations of treatments with ethanol, commercial bleach and mercuric chloride with sodium lauryl sulphate failed to produce many apparently uncontaminated cultures. In Hammatt (1994) it is noted that shoots of a mature clone were contaminated with a species of *Bacillus* bacteria that only grew on the plant material possibly alluding to a similar coexistence *in vivo*. In Hammatt's (1997) trial only two cultures were obtained from 120 initial explants of one genotype and one culture from 23 initial explants of another.

Heat treating ash buds at 38°C for 12 hours was used to break bud dormancy in apical explants by Silveira and Cottignies (1994), and according to Langens-Gerrits *et al.* (1998) can also been used to decontaminate plant tissues. However, it was noted in our experiments that after heat treatment some explants turned brown and died. Tissues damaged by heat treatment may have a more severe wounding reaction when the explants are cut thus the production of volatile compounds that occurs after wounding (e.g. ethylene and ethane) as well as phenolics may increase (Langens-Gerrits *et al.*, 1998).

In order to reduce the explant browning problem experienced in all of the experiments involving heat treatment of the explants, dormant apical buds were treated at a higher temperature (45°C) for a shorter time period (one hour) then cultured with or without light. The viability of dormant apical buds was improved by heat treatment followed by culturing in the light. This result was consistent over two years, 2002 and 2003 in genotype 5, and for wild genotypes in 2003. Placing the explants in dark conditions after heat treatment had a negative effect on viability.

Langens-Gerrits *et al.* (1998) reported a successful reduction of contamination after heat treating dormant winter buds of *Acer lobelia* at 40 – 45°C compared to controls receiving no heat treatment, furthermore, the heat treatment had no adverse effect on bud viability. George and Sherrington

(1984) mentioned the possibility of preventing or reducing the activity of enzymes concerned with both the biosynthesis and oxidation of phenolics, by keeping the cultures in the dark. Browning was eliminated in apical and nodal explants of *Pistacia vera* L. cv. mateur and *Pistacia atlantica* Desf. by adding activated charcoal to the medium combined with dark periods of 4 – 8 days (Mederos-Molina and Trujillo, 1999). Pittet and Moncousin (1982) attribute the reduction of explant browning by placing shoots in darkness to the fact that polyphenol oxidase activity is light induced. In our experiments, culture dishes in the dark post-heat treatment environment were enclosed in a cardboard box to exclude the light and an explanation for the low viability rates maybe that during and after heat treatment metabolism, and therefore the need for oxygen, increases (Langens-Gerrits *et al.*, 1998). During heat treatment in the water bath the tissue is under partial anaerobsis because it is submerged (Baker, 1962b). Oxygen deficiency may lead, for example, to production of acetaldehyde and ethanol (George, 1993). Accumulation of acetaldehyde and ethanol was followed by rapid tissue deterioration in *Prunus* (Righetti *et al.*, 1990). Thus, death of the explants may have been caused by asphyxiation of the tissue resulting in an accumulation of acetaldehyde and ethanol. This phenomenon was seen in *Lilium speciosum* bulbs heat treated at 43°C for one hour in the water bath and placed in hermetically closed tubes (Langens-Gerrits *et al.*, 1998). Furthermore, occasionally, it has been found that after heat treatment contamination rates increase, this may be due to remaining pathogens being easily released from damaged tissue or that the high temperatures activate spores of pathogens present in the tissue (Langens-Gerrits *et al.*, 1998).

Viability was significantly increased in heat treated apical buds cultured on medium supplemented with the antioxidant glutathione (GSH) (5mg/l). When the experiment was repeated in 2003, the results were compromised due to excessive contamination, however it was noted that there were less explant deaths due to browning on the media supplemented with 1mg/l glutathione. The positive effects of the GSH treatment indicate that it is possible to control the browning of the explants using GSH. Inclusion of antioxidants into the culture medium can often prevent browning (Herman, 2000). Citric acid, dithiothreitol, and 2-mercaptoethanol are commonly used as biological antioxidants. However, it is believed that the key reductant in living cells is glutathione, which is very abundant (Kosower and Kosower, 1978). Therefore, of the antioxidants used so far, GSH is believed to be the least toxic and the most suitable (Nomura *et al.*, 1998). Glutathione is a natural antioxidant known to relieve stresses such as oxidation and believed to play a role in the defence system of plants (Herman, 2000). Reduced glutathione (GSH) is thought to be an ideal antioxidant because it should be ubiquitous in cells and easily metabolised (Nomura *et al.*, 1998), furthermore it has also been suggested that glutathione has a role in overcoming dormancy in plants (Fuchigami and Nee, 1987). A drop of GSH solution placed on olive (*Olea europaea*) callus in the dark reduced browning (Ruigini, 1984). Nomura *et al.* (1998) applied GSH to prevent browning of shoot tip explants of apple (*Malus pumila* Mill.) their findings suggest that a dip treatment in GSH at

the initial phase of culture is more effective for normal shoot development compared to a dip treatment combined with GSH added to the culture medium which promoted callus growth.

The most successful method of initiating explants *in vitro* from dormant winter material proved to be from apical bud derived shoots produced by forcing stem cuttings, as described in chapter 2. Axillary bud derived shoots used for culture initiation were significantly less viable *in vitro*. To yield more hygienic explants, stems of ash can be collected from field grown material, then heat treated to break dormancy and reduce contamination. The technique of forcing buds on severed defoliated shoots (without heat treatment) is very useful for obtaining out of season explants from dormant material (Skirvin *et al.*, 1986; Vieitez *et al.*, 1986; Chalupa, 1996). According to George (1993) and Skirvin *et al.*, (1986) not only are the explants from forced dormant buds relatively contaminant-free but they may also be more readily established in culture than explants taken during spring and summer, and less prone to polyphenol exudates. Skirvin *et al.*, (1986) found that forced dormant winter buds of *Malus x domestica* Borkh. provided the best explants for initiating cultures. Vieitiz *et al.*, (1986) used forced dormant shoots of *Castanea sativa* x *C. crenata* for initiating cultures and Chalupa (1996) initiated cultures from forced shoots of *Fagus sylvatica* L. Marks *et al* (1987) used the forcing method for initiating cultures of *Quercus robur* 'Fastigiata', for various *Acer* species and for clones of *Crataegus* however, it was found that varieties of plants responded to treatment differently. Perez-Parron *et al* (1994) successfully established cultures from mature *Fraxinus angustifolia* by forcing shoots in distilled water alone or with the growth regulators BA, 1-naphthalene acetic acid (NAA) or GA_3. It was observed that but sprouting was not improved by growth regulators and may even be inhibited by GA_3 and NAA.

The reason for the increased viability of the apical bud derived shoots produced by forcing methods might be explained by the control of apical dominance over the growth of axillary buds. Axillary buds are held in a state of quiescence until their release from inhibition, which can occur very quickly after decapitation. IAA is believed to play a major role in apical dominance via interaction with cytokinins. Cytokinins are synthesised at various meristematic sites in the plant, including shoot apex, young leaves, cambial region and most importantly in the root apices (Srivastava, 2002). Cytokinins synthesised in the roots are transported upward into the shoot via the xylem stream and release the lateral buds from inhibition. The lateral buds are kept repressed by a certain ratio of cytokinin to IAA and this ratio can change in favour of cytokinins by a cut at the apical end. Decapitation experiments indicate that reduced auxin levels after decapitation may induce bud outgrowth by causing a transient increase in cytokinin content (Bangerth, 1994; Li *et al.*, 1995). However, the stem cuttings used in the experiments in this chapter had no roots therefore no transport of endogenous cytokinin could take place from the roots. The presence of developed roots does not appear to be a prerequisite for bud inhibition or lateral shoot growth in the initial stages (Hillman, 1985). However, for further growth and elongation of the shoot the presence of cytokinin, supplied by the roots, and reserves from the stem may be required. It has been shown in

the ancestral potato *Solanum andigena*, the transformation of stolons into normal orthogravitrophic shoots following shoot decapitation requires the presence of roots or cytokinins (Woolley and Wareing, 1972a; Woolley and Wareing, 1972b).

In addition, from the experiments on forced shoots it was apparent that the older apical bud derived shoots which were harvested at six weeks after heat treatment were more viable *in vitro* compared to younger shoots harvested three weeks after heat treatment. Geneve *et al.* (1992) initiated cultures of Eastern Redbud (*Cercis Canadensis* L.) *in vitro* by selecting vigorous shoots 30-40mm long as explants from forced dormant shoots. There is frequently an optimum size for explants used to initiate tissue cultures, and from the results in this thesis it was determined that the older apical shoots which were generally greater than 10mm in length were more likely to survive *in vitro*. Very small explants, whether they are shoot tips, fragments of whole plant tissues or pieces of callus, do not survive well in culture, but large explants may be more difficult to decontaminate (George, 1993). According to George (1993) large explants have an advantage over smaller ones for initiating shoot cultures in that they:

- better survive the transfer to *in vitro* conditions,
- more rapidly commence growth, and
- contain more axillary buds.

During the experiments, warm water treatments were not applied to the summer shoots as it was feared that the tissue would be severely damaged in the soft green shoots. Pre-sterilisation treatments such as washing in running water and dipping in 70% ethanol were tested and found to be necessary to produce clean explants for culture initiation (section 3. 8. (1. 2)). Bellarosa (1989) advises to wash all explants thoroughly under running water to eliminate earth, dead tissues and insect eggs before passing to the sterilisation phase. According to George (1993) washing in water containing a wetting agent and/ or washing in running water helps reduce the level of superficial contaminants and makes the organisms more liable to be killed by subsequent treatments with disinfectants. Pre-sterilisation treatments, such as a dip in 70% ethanol, is widely used in plant tissue culture (George, 1993) and has been used during sterilisation of mature *Fraxinus excelsior* by Pierik and Sprenkels (1997) and in mature *F. ornus* (Arrillaga *et al.*, 1992), seeds of *F. pennsylvanica* (Kim *et al.*, 1997) and *F. angustifolia* (Tonon, 2001a). Alcohols are not only germicidal, but also remove surface waxes from plant tissue, thus a preliminary dip in ethanol permits plant tissues to be more effectively wetted and penetrated by another germicide (George, 1993). The results of experiments in this chapter show that a post sterilisation dip in 100mg/l copper sulphate, a well known fungicide, reduced contamination significantly compared to the control treatment (table 3. 3). However, it is important to optimise the correct concentration of $CuSO_4$ as too high a concentration can be toxic to plants as well as to fungi (Bowen, 1979).

When initiating shoot cultures from naturally flushing summer shoots it was discovered that nodal explants were more likely to survive *in vitro* than apical explants, this was true for all three genotypes tested (table 3. 7). Several reports of culture initiation involving *F. excelsior* report success with apical explants (Silveira and Cottignies, 1994; Hammatt, 1994). Chalupa (1990) used apical and nodal explants taken from greenhouse grown seedlings but failed to give any data on success rates from either type of explant. Arrillaga *et al* (1992) established shoot cultures of mature *F. ornus* from apical buds. Cultures have been initiated from mature olive trees (*Olea europaea*) from nodal explants (Rugini, 1984; Santos *et al.*, 2003). The rationale determining which bud is used as an explant to initiate a culture has received little attention in the literature. Welander (1988) isolated both apical and axillary bud explants of *Betula pendula*, and showed that these displayed different cultural and biochemical characteristics *in vitro* as determined by their position on the plant. The inhibitory influence of the apical bud on the axillary bud is removed *in vitro* and the buds can fully express their growth potential. Welander (1988) reported stronger growth *in vitro* from resting buds of dormant plants, and attributed this to the accumulation of starch and soluble carbohydrates in these buds.

The optimum media for initiating explants from naturally flushing shoots of mature ash in this study was found to be modified WPM medium when compared to M91 medium (containing modified MS salts and plant growth regulators) (table 3. 13). *Fraxinus excelsior* has been previously initiated on various different media including MS, WPM, QL and DKW (Hammatt and Ridout, 1992; Silveira and Cottignies, 1994). Both these authors agree that the MS medium gave the least satisfactory results, the former reporting that shoots grown on MS medium became chlorotic and the latter reporting a high proportion of explant deaths. WPM medium has a lower ionic concentration than MS, DKW or QL media. It has been shown with forest trees that weaker formulations promote axillary bud development (McCown and Sellmer, 1987). Different culture media have been used for different olive cultivars, indicating differences in adaptability and requirements of *in vitro* cultures (Grigoriadou *et al.*, 2002). Both Hammatt (1994) and Silveira and Cottiginies (1994) established ash cultures *in vitro* without the use of plant growth regulators, only using them to initiate shoot production. As early as 1969, Nitsch and Strain demonstrated that cytokinins, which are N^6-substituted adenines, enhanced browning due to the oxidation of phenolics produced by the plant. Therefore it is recommended to omit this type of cytokinin when initiating cultures (Debergh and Read, 1991). Chalupa (1990) reported that the concentration of external cytokinin had the greatest effect on shoot growth and that media containing high levels of cytokinin (BAP 2.0 - 5.0 mg/l) and low levels of auxin (IBA 0.1 mg/l) with thidiazuron (0.01 mg/l) gave the best shoot growth, M91 medium is a similar medium and is used as standard for maintaining ash cultures *in vitro* at the micropropagation laboratory in the Teagasc Kinsealy Research Centre (Douglas, personal communication).

The viability of nodal explants from naturally flushing shoots was significantly reduced by supplementing the initiation media with the organic nutrient adenine sulphate and the antioxidant ascorbic acid (table 3. 14). Both ascorbic acid and adenine sulphate were added to MS medium when propagating nodes from mature a Khair tree (*Acacia catechu* Willd.) (Kaur *et al.*, 1998). Adenine in the form of adenine sulphate is believed to stimulate cell growth and enhance shoot formation (Thom *et al.*, 1981). It provides an available source of nitrogen to the cell and can generally be taken up more rapidly than inorganic nitrogen (Thom *et al.*, 1981). Interestingly, Hammatt (1997) suggests that the performance of ash cultures on WPM could be improved by adding more nitrogen to the low salt media. However, adenine sulphate does not always give beneficial results and was found to inhibit the formation of shoots cultured from tuber discs of potato (Jarret *et al.*, 1980). Ascorbic acid, also known as vitamin C, is synthesised by plants and occurs naturally in cells in relatively high concentrations (George, 1993). Ascorbate acts as a reductant, being converted by oxidation to dehydro-ascorbic acid. Ascorbic acid works as an antioxidant by scavenging injurious hydrogen peroxide and some other activated oxygen radicals (Foyer *et al.*, 1983; Thompson *et al.*, 1987). While inclusion of antioxidants in the culture medium can often prevent browning, antioxidants can be toxic to the explant (Herman, 2000).

Explants taken from previously micropropagated plants transferred to the greenhouse showed the highest viability levels and were easily established *in vitro* (section 3. 8. (3. 2)). This may be due to the re-invigoration of the plants during their *in vitro* propagation. In a number of plant species repeated subculture of adult shoots results in re-invigoration or complete rejuvenation, by which shoot multiplication and rooting ability are strongly improved (Pierik, 1990). Explanations given for rejuvenation are often vague and speculative, since very little is known about the physiological and biochemical factors causing rejuvenation (Hackett, 1985). Hammatt (1994) reported the apparent re-invigoration of a mature ash clone *in vitro,* it was noted that shoots became progressively easier to root with successive subcultures. Re-invigoration of mature woody plants via *in vitro* culture has been reported for such species as *Vitis vinifera* (Mullins *et al.*, 1979), hybrid *Betula platyphylla* var. *japonica* x *B. pendula* (Meier-Dinkel, 1991) and *Larix decidua* (Kretzschmar and Ewald, 1994). Cultures from mature species are frequently initiated from material with juvenile characters such as stump sprouts and epicormic shoots (Vieitez *et al.*, 1985; Vieitez *et al.*, 1986; Meier-Dinkel, 1987; San-Jose *et al.*, 1988; Chalupa, 1993).

Contamination levels in the explants varied between years. By examining the data collected over the two years, 2002 and 2003, it was observed that for the winter buds contamination levels were less in 2002 than in 2003 (table 3. 4). However, the opposite is true for the summer shoots, which showed contamination levels of over 90% in 2002 compared to between 55-96% contamination in 2003 (table 3. 10). The lowest contamination levels for naturally flushing shoots were recorded from explants sourced in November 2001 from micropropagated mother plants kept in the greenhouse (table 3. 15). In both 2002 and 2003, contamination levels were lowest for explants

taken from flushed summer shoots earlier in the season compared to those taken as the season progressed (table 3. 10). This was also seen in the dormant winter buds in 2002, where contamination was lowest for explants taken in January compared to those taken in March (section 3. 8. (2. 1.ii)).

Generally, cultures are established most readily from explants gathered at the start of, or during, active growth of the mother plant, but the best time of year for sampling may depend on the kind of culture to be grown, on the particular genotype to be used, and on the relative amounts of contamination and browning of the explant occurring at each season (George, 1993). In temperate climates contamination almost invariably increases as the growing season progresses and shoots of many plants are less contaminated when they are in active growth than at other times (George, 1993; Chalupa, 1996). By growing mother plants in the greenhouse they are kept in clean conditions with no overhead irrigation thus minimising contact with pathogens. It is commonly accepted that plants growing in the field are subjected to high rates of contamination and a variable environment (George, 1993).

Bud viability was also dependant on the month of culture initiation. Dormant apical shoots of selected genotype 7 showed higher viability rates in January compared to February or March when given the same heat treatment. In 2002 it was demonstrated that July was the optimum month during the summer season to initiate explants *in vitro* and no explants survived when initiated in August. In 2003 there was no significant difference in viability between May, June and July, however, viability decreased from 24.6% in May to 14.5% in July.

Dormant winter buds used as explants for culture initiation by Silveira and Cottignies (1994) produced sprouts when harvested in September, January and March whereas similar explants harvested during the growing season failed to grow. As mentioned previously forced dormant shoots of *Malus x domestica* provided the best explants for initiating cultures (Skirvin *et al.*, 1986). Vieitiz *et al.*, (1986) used forced dormant shoots of *Castanea sativa* x *C. crenata* for initiating cultures. Cottignies (1986) showed that ash apical bud explants, harvested during the resting period, possess stored energy sources such as starch, which can be used immediately for growth recovery. In contrast, in the active apical buds, a lack of such resources could impede cell proliferation in the explant (Silveira and Cottignies, 1994). Studies on several species of *Prunus* (Quoirin and Lepoivre, 1977), *Populus (*Beauchesne and Poulin, 1972*)*, *Fagus sylvatica* (Nadel *et al.*, 1991) and *Picea sitchensis* (Selby and Harvey, 1985) have shown that the resting period of growth is a favourable time to take explants for *in vitro* culture. As mentioned previously Silveira and Cottignies (1994) recommended heat treating buds at 38°C to break dormancy and that January was the optimum month to harvest the explants. Warm water treatment can damage the host plant (Baker, 1962a) and the resistance of plant tissue depends on the physiological condition, for example size, moisture content, vigour, condition of external layers, temperature conditions

during growth, dormancy level, age and genetic constitution (Baker, 1962a). Wisniewski *et al.* (1994 and 1997) found that thermotolerance in hybrid poplar increased during endodormancy (October to January) and decreased during the latter part of ecodormancy (March). They found that as the season progressed and the buds were no longer endodormant, temperatures which had previously induced the highest percentage bud break (40 - 45°C), were resulting in inhibition of bud break and high bud mortality indicating a shift in the thermotolerance of the buds. In temperate plants where shoots are newly initiated each year from resting buds, shoots can be said to become progressively older as the season advances (George, 1993). Explants for shoot tip or meristem culture are best dissected from young shoots (Kaul, 1986). Skirvin *et al.* (1986) and Chalupa (1996) found that flushing shoots collected early in the season responded better to *in vitro* establishment than those collected later in the season.

The viability of the ash explants *in vitro* was greatly dependant on their genotype as illustrated in experiments involving both dormant winter buds and flushed summer shoots. When the effect of a warm water treatment on different genotypes of *F. excelsior* was tested, the genotypes responded very differently, with viability rates ranging from 65% for genotype 5 to 0% for genotype 49 in 2002, even though contamination rates were similar (table 3. 4). Indicating differences in thermotolerance perhaps due to genetic differences, age and/ or dormancy level. In experiments involving flushed summer shoots the viability of different explant types *in vitro* varied significantly between genotypes (table 3.7). All three genotypes tested were more successfully initiated from nodal explants compared to apical explants, genotypes 7 and 49 could only be initiated from nodes and the viability of nodal explants varied significantly between genotypes. The highest viability rates *in vitro* were for explants taken from previously micropropagated mother plants. The fact that those genotypes were already successfully established *in vitro* may indicate that they are more susceptible to initiation and *in vitro* survival.

According to George (1993) the growth of cultured tissues *in vitro* is more influenced by genotype than by any other factor. There are numerous examples in the literature in which the results obtained during tissue culture have varied from one variety of plant to another, and it is probably true to say that the effects of genotype impose one of the greatest constraints to the tissue culture and micropropagation of plants (George, 1993). In *Eucalyptus marginata*, for example, the capacity of shoot tip explants from different mature mother plants to grow *in vitro* showed large differences, as did the viability of newly explanted shoot tips from different peach cultivars (Hammerschlag, 1982), and from different clones of sessile oak (*Quercus petraea* (Matt.) Liebl) (San-Jose *et al.*, 1990). According to George (1993) the genetic control of growth and morphogenesis *in vitro* can result from either the primary or secondary effect of genes. Thus certain kinds of morphogenic responses in tissue cultures are seen to be directly related to the behaviour exhibited by whole plants or parts of plants *in vivo*, whereas features of the whole plant which influence other characters of *in vitro* culture, such as callus production are more difficult to determine. Genetically

determined characteristics of the latter kind probably result from only the secondary effect of normal gene function. This represents a spurious pleiotropy in which the main effect of a gene or genes causes other unrelated things to happen.

In conclusion, the experiments in this study have shown that explants from mature *F. excelsior* can be initiated *in vitro* from stem cuttings of dormant winter buds forced in the greenhouse, from summer shoots collected early in the season and from previously micropropagated plants weaned to greenhouse. The main difficulties experienced were decontamination of the explants and explant browning. Explants from previously micropropagated plants were the most viable *in vitro*, followed by summer shoots collected from greenhouse grown grafted plants. This may be partly due to the hygienic conditions of the greenhouse compared to that of the field. The optimum decontamination procedure for explants from flushed shoots was a wash in warm water and teepol® followed by washing in running water prior to sterilisation. Initiating explants from the grafted plants was best done on modified WPM medium with no supplements. All the experiments involving explants from mother trees of different genotype showed large differences among the observed responses, lending further support to the notion that the response to *in vitro* culture depends heavily on the genotype of the stock plant.

CHAPTER 4: Shoot Production

4. 1. Introduction

The objective of the shoot production or proliferation phase of micropropagation is to bring about the production of new plant outgrowths or propagules, which when separated from the culture are capable of forming intact plants (George, 1993). For the purposes of this project, a reproducible method was developed, suitable for a range of *F. excelsior* genotypes and that would limit risk of introducing somaclonal variation into the culture line. In order to achieve this task certain areas of the shoot production stage had to be optimised; this included the culture regime, culture media nutrient composition, carbohydrate source and plant growth regulator concentration.

4. 2. Axillary shoot proliferation

Axillary shoots are those that originate in the axils where leaves are attached to stems. This is the normal location where shoots are expected to form on a plant, and such shoots have a direct lineage to the shoot apical meristem in the seed. When a shoot forms on a plant at a location that is other than a node, for example from internodes, leaves, roots or callus, this is not normally expected and these shoots are considered to be adventitious (Preece, 1997). An advantage of axillary shoot proliferation over the production of adventitious shoots for micropropagation is the reduction in somaclonal variation with axillary shoots. Axillary shoots form from preformed meristems at nodes and the chances of DNA in the organised shoot meristem undergoing a mutation is relatively low (Preece, 1997). Therefore, axillary shoot proliferation is the preferred method to propagate plants clonally.

4. 3. Culture regime

Culturing explants on media containing plant growth regulators for extended periods can lead to certain physiological disorders such as vitrification, shoot fasiation and excessive callus production (George, 1993; Hutteman and Preece, 1993; Ziv, 1991). Alternating this media with one containing activated charcoal may be beneficial to the shoots as activated charcoal can negate the effects of plant growth regulators by absorbing them and any other inhibitory substances accumulating in the culture medium (Fridborg *et al.,* 1978; Weatherhead *et al.,* 1978, 1979; Ebert and Taylor, 1990). According to Krikorian (1995), it is sometimes helpful to pulse a system intermittently with cytokinin between subcultures to modulate the branching pattern, since too high a level of cytokinin can induce genetic change and too low a level may not stimulate axillary bud break. In plants where a uniform single stem is preferred, such as forest trees, a system may profit by alternating culture on a high cytokinin medium and a cytokinin free medium that allows stem elongation. Jona and Vigliocco (1985) added charcoal to the medium on which *Prunus persica* shoots were to be elongated, finding that it overcame the negative effects of the high rate of cytokinin previously used in shoot proliferation.

4. 4. Polyamines as indicators of plant stress *in vitro*

In this study, the response of *in vitro* cultures of *F. excelsior* in three different culture regimes was assessed by measuring the levels of the endogenous polyamines putrescine, spermine and spermidine in the leaves. In plants, the biologically ubiquitous polyamines (PAs) are implicated in the control of growth and developmental processes (Bagni, 1989; Galston and Kaur-Sawhnet, 1995; Kumar *et al.,* 1997) as well as in the response to a wide range of stress conditions, such as mineral nutrient deficiencies, osmotic stress, salinity, low external pH, temperature stress, hypoxia, atmospheric pollutants or infection by pathogens (Bouchereau *et al.,* 1999; Flores 1991; Flores and Martin-Tanguy, 1991).

Polyamines are low molecular weight, strongly basic molecules found ubiquitously in all living organisms, bacteria, fungi, animals, and plants studied so far (Srivastava, 2002). In plant cells the diamine putrescine [$H_3N^+(CH_2)_4\ NH_3^+$], triamine spermidine [$H_3N^+(CH_2)_3\ NH_2^+(CH_2)_4\ NH_3^+$] and tetraamine spermine [$H_3N^+(CH_2)_3\ NH_2^+(CH_2)_4\ NH_2^+(CH_2)_3\ NH_3^+$] constitute the major PAs. In both prokaryotes and eukaryotes (Tabor and Tabor, 1984), including higher plants (Evans and Malmberg, 1989), mutants lacking the ability to biosynthesize PAs are unable to grow and develop normally, since the addition of PAs to these mutants generally restores normal growth and development, it is reasonable to conclude that PAs are essential to all cells (Galston and Kaur-Sawhney, 1995).

PAs are synthesised in plants in two major steps (Srivastava, 2002). Putrescine is synthesised from arginine or ornithine via arginine decarboxylase (ADC) or ornithine decarboxylase (ODC), respectively. Spermine and spermidine are synthesised from putrescine by successive transfers of aminopropyl groups from S-adenosylmethionine (SAM). For such a transfer, SAM is first decarboxylated by SAM decarboxylase, and the aminopropyl groups are attached to spermidine or spermine via specific synthetases. ADC and ODC can be inhibited irreversibly by DL-∝-diflouromethylarginine (DFMA) and DL-∝-diflouromethylornithine (DFMO), respectively. Methylglyoxal-*bis*-guanyl hydrazone (MGBG) and cyclohexylamine are reversible inhibitors of SAM decarboxylase and spermidine (or spermine) synthetase activities, respectively.

At cellular pH, PAs are polycations and thus bind readily to such important cellular polyanions as DNA, RNA, phospholipids and acidic protein residues and cell wall components. Through such binding, PAs could affect the synthesis of macromolecules, the activity of macromolecules, membrane permeability, and partial processes of mitosis and meiosis (Galston and Kaur-Sawhney, 1995). Polyamines have been variously implicated in regulation of cell division, embryogenesis, pollen formation, fruit development, breaking of dormancy, internode elongation, root formation, senescence, and protection against environmental stresses (Galston, 1983; Faust and Wang, 1992). The postulated role of polyamines is based on their ubiquitous distribution in plant cells; their changes in response to stimuli such as light, growth hormones, and environmental stresses

(e.g. drought, low temperature, mineral deficiency) and their effects on plant morphogenesis and growth when applied exogeneously (Kolzowski and Pallardy, 1997a). RNA and DNA are stabilised by their association with PAs, and in their bound form are more resistant to nuclease enzymes and thermal denaturation (Galston *et al.,* 1980). Free polyamines can compensate for ionic deficiencies or stress within plants, and can temporarily substitute for, and sometimes have the same physiological effect as, the cations K^+, Ca^{2+} and Mg^{2+} (George, 1993). Part of their function may be to act as buffers to minimise fluctuations in cellular pH (Slocum *et al.,* 1984). The concentration of putrescine is high in plants grown in conditions which favour the production of H^+ ions, such as acid stress, or where ammonium ions are provided as the major source of nitrogen (George, 1993). The modulation of growth caused by these compounds probably springs from their ability to bind to phospholipid groups and other anionic sites on membranes, cell wall polysaccharides, and nucleic acids such as DNA, tRNA and mRNA. They are thought to modulate enzyme activities, enhance DNA replication and transcription, and to be necessary to convert the structure of tRNA from an inactive to active form (Bagni and Biondi, 1987). Levels of polyamine compounds tend to be highest in actively growing tissues and organs, such as root tips, buds (Kulpa *et al.,* 1985) and cells and tissues undergoing, or which have just undergone, morphogenesis (Tiburcio *et al.,* 1987; Wang and Faust, 1986; Martin-Tanguy *et al.,* 1988).

4. 5. Culture media nutrient composition

The chemical composition of the culture medium plays an important role in the success of micropropagation (Nas and Read, 2004). A sub-optimal culture medium may cause physiological disorders or death of tissue (Nas and Read 2000; Preece, 1995). Chemical composition of the culture medium has been reported to affect all types of morphogenic responses, including caulogenesis (*de novo* shoot production) (Morard and Henry, 1998), axillary bud proliferation (Schnapp and Preece, 1986), plant regeneration (Sahrawat *et al.,* 1999) and embryogenesis (Gyulai *et al.,* 1992). Nutrient salts and plant growth regulators have been known to interact in the culture medium, and it is generally accepted that to obtain the most effective results from growth regulator treatments, it is usually important to see that the nutritional components of the medium are at the most favourable levels (Murashige and Skoog, 1962; De Fossard *et al.,* 1974; George, 1993). Some common culture media formulations used for woody plant culture including that of *Fraxinus* species, and tested in this project for *F. excelsior*, are described below.

Murashige and Skoog (1962) medium formulation (MS medium) is the most widely used tissue culture medium and many variations have been developed. The medium is derived from White's medium (White, 1943) and was originally developed for the cultivation of *Nicotiana tabacum* calli. Compared to White's medium, MS medium has higher concentrations of all nutrients, especially the macronutrients nitrogen and potassium. Due to the high concentration of minerals, MS medium is a very rich saline medium and can be too salty to certain plant species, including *Quercus robur* and *Q. petraea* (Chalupa 1984, 1993; Vieitez *et al.,* 1985; Meier-Dinkel, 1987; Meier-Dinkel *at al.*,

1993). The original MS medium vitamins are sometimes replaced with Gamborg *et al.,* (1968) B5 medium vitamins which have a higher concentration of vitamins especially thiamine which is important for plant growth (George, 1993).

Lloyd and McCown (1980) developed "Woody Plant Medium" (WPM medium) for the shoot culture of trees and shrubs such as *Betula*, *Kalmia*, *Rosa* and *Rhododendron* (Lloyd and McCown, 1980, 1981; McCown and Llyod, 1981). Since then this medium has been widely used for the propagation of ornamental trees and shrubs. It is characterised as being a low nutrient medium and differs from MS medium by having calcium nitrate and potassium sulphate instead of potassium nitrate.

Driver and Kuniyuki (1984) developed DKW medium for the *in vitro* propagation of Paradox walnut rootstock (*Julgans hindsii x J. regia*). Since then this medium has been used to propagate many woody species including *Corylus avellana* L. (Yu and Reed 1993, 1995). DKW medium is characterised as having a higher nutrient concentration than WPM medium and in particular a high Ca^{2+} ion concentration in comparison to both WPM and MS medium.

Ruigini (1984) formulated olive medium (OM) by analysis of the mineral elements in embryos and young shoots of *Olea europaea* found to be characterised by a high content of Ca, Mg, S, Cu and Zn hence, the OM proliferation medium was formulated with higher levels of these elements compared to MS medium. Furthermore, the vitamins folic acid and biotin are included in OM medium. Biotin is an essential cofactor for a variety of carboxylase and decarboxylase reactions and is involved in diverse metabolic pathways of all organisms (Wood and Kumar, 1985), folic acid has been shown to have a growth-stimulatory effect on cultured plant tissues (de Capite, 1952). The medium has since been used to propagate Oleaceae species such as *Fraxinus ornus* (Arrillaga *et al.,* 1992) and *Phillyrea latifolia* L. (Lucchesini and Mensuali-Sodi, 2004).

Nutrient requirements are an important consideration for *in vitro* culture of *Fraxinus*. Chalupa (1990) reports success with culturing *F. excelsior* on both MS and DKW medium. Both Silveira and Cottignies (1994) and Hammatt and Ridout (1992) report the greatest success culturing *F. excelsior* on WPM and DKW medium, respectively. Pierik and Sprenkels (1997) reported equal success culturing *F. excelsior* on either WPM salts or MS salts at 0.75 strength. None of these authors, however, report having tested Ruigini's OM for culturing *F. excelsior*. Other species of *Fraxinus* including *F. americana* (Preece *et al.,* 1987; Preece *et al.,* 1989) and *F. angustifolia* (Perez-Parron *et al.,* 1994) are best propagated using either WPM or DKW medium. As mentioned previously, OM media has been used for *in vitro* propagation of *F. ornus* (Arrillaga *et al.,* 1992). The earlier work has shown that various *Fraxinus* species may possess unique nutritional requirements, as *F. americana*, *F. excelsior* and *F. ornus* exhibit suitable axillary proliferation on MS, DKW and OM media, respectively (Arrillaga *et al.,* 1992; Hammatt and Ridout, 1992; Navarrete *et al.,*, 1989).

4. 5. 1. Carbohydrate source

The responses of *in vitro* cultures to different carbon sources added to the medium are frequently tested during medium optimisation for various species. Sucrose is almost universally used for micropropagation purposes as it is so generally utilisable by tissue cultures (George, 1993). Some of the first work on the carbohydrate nutrition of plant tissue identified sucrose as the best source of carbon followed by glucose, maltose and raffinose (Gautheret 1945). Glucose is generally found to support growth equally well, and in a few plants it may result in better *in vitro* growth than sucrose; but being more expensive than sucrose, glucose will only be preferred for micropropagation where it produces clearly advantageous results (George, 1993). Pierik and Sprenkels (1997) tested both glucose and sucrose for the *in vitro* culture of *F. excelsior* and found that sucrose was the better carbohydrate to support the growth and elongation of shoots.

Presumably the most suitable support for *in vitro* growth is provided by carbohydrates which the plant produces and transports for long distances (Leifert *et al.,* 1995). Many plant species, including members of Oleaceae, produce mannitol as their main transport carbohydrate (Stoop *et al.,* 1996; Peltier *et al.,* 1997). Mannitol and other polyols (sugar alcohols), such as sorbitol have several functions in the plant other than translocation and storage of carbon such as, osmotic adjustment, cryoprotection, prevention of activated oxygen species, boron transport, and energy delivery, has been proposed (Brown and Hu 1996, Escobar-Gutierrez and Gaudillbre 1996). Mannitol has been included in culture medium used to propagate *O. europaea* (Leva *et al.,* 1994), *P. latifolia* (Lucchesini and Mensuali-Sodi, 2004) and *Apium graveolens* (Vítová *et al.,* 2002) all of which use mannitol as a transport carbohydrate *in vivo*. Degradation of mannitol could be expected to be even more efficient from an energetic point of view, when compared with the metabolism of sucrose, as nicotinamide adenine dinucleotide (NADH) is produced in the initial step of mannitol catabolism and can be oxidised in mitochondria producing a theoretical yield of an additional three adenine tri-phosphate (ATP) molecules (Pharr *et al.,* 1995). Due to the nature of sugar alcohols, such as mannitol and sorbitol, as compatible solutes, they are involved in the osmotic adjustment of cells and thus play an important role under osmotic stress (Brown and Simpson, 1972). It is important to test all carbohydrates at equimolar concentrations because when different carbohydrates are added using the reference in weight per volume (w/v) or percentage rather than molar concentration, an osmotic variable that may mask the explant response is introduced into the treatments (De Paiva Neto and Otoni, 2003).

4. 5. 2. Plant growth regulators

Plant growth regulators are synthetic chemicals that have a regulatory rather than a nutritional role in plant growth and development. The effects of growth regulators are generally not absolute and specific (George, 1993). The responses of cells, tissues and organs *in vitro* can vary according to cultural conditions, the type of explant and the genotype of the plant. Cytokinins are plant growth regulators frequently used in tissue culture where they stimulate cell division and control

morphogenesis (Biondi and Thorpe, 1982; Villalobos *et al.*, 1984). Natural cytokinins such as 2-ip and zeatin can be used in research but tend not to be employed in commercial laboratories routinely due to their cost. Fortunately, several chemical analogues of natural cytokinins apart from kinetin, which is not now thought to be a naturally occurring cytokinin (Hecht, 1980), have been prepared which are found to be highly active as cytokinins (George, 1993). Benzyladenine (BA, or BAP) is commonly used for *in vitro* culture of woody species including *Prunus armeniaca* L., *Julgans nigra* L., *Artocarpus heterophyllus* Lam. (Murai *et al.*, 1995; Driver and Kuniyuki, 1984; Roy *et al.,* 1990). Many authors have also used BA for micropropagation of *F. excelsior* including Chalupa (1990), Hammatt and Ridout (1992), Hammatt (1994) and Silveira and Cottignies (1994).

Frequently, shoot proliferation can be significantly enhanced by the addition of a low concentration of auxin or by adding a second cytokinin to the proliferation medium. According to Huetteman and Preece (1993) explants of some woody species (especially trees), having naturally strong monopodial growth habits, may not branch *in vitro* sufficiently when using the more common amino purine cytokinins, thidiazuron (TDZ) offers an alternative that frequently enhances shoot production of these species. Cultures of *Robinia pseudoacacia* L., *Sorbus aucuparia* L., and *Tilia cordata* Mill. increased in shoot proliferation and elongation when BA plus IBA or NAA were added to a TDZ containing medium (Chalupa, 1987). Chalupa (1990) used BA in combination with TDZ for micropropagation of *F. excelsior*.

TDZ is used for micropropagation of a wide array of woody species because of its tremendous ability to stimulate shoot proliferation (Huetteman and Preece, 1993). It is a potent stimulator of shoot organogenesis in woody species, including *F. americana* (Bates *et al.,* 1992), *Malus* (Fasolo *et al.,* 1989), *Prunus* (Mante *et al.,* 1989), *Rhododendron* (Preece and Imel, 1991) and *Rubus* (Fiola *et al.,* 1990). In most of these cases TDZ was more potent than, or equal to, aminopurine cytokinins for stimulating shoot organogenesis. Thidiazuron was developed originally by Schering AG for utilisation as a defoliant for cotton (*Gossypium hirsutum* L.) (Arndt *et al.,* 1976). Mok *et al.,* (1982) found that cytokinin-dependant *Phaseolus lunatus* L. callus was stimulated to grow when low concentrations of TDZ were added to an *in vitro* medium. Thidiazuron was much more active than zeatin for stimulating the growth of *Phaseolus* callus. Compared to most other active compounds added to the culture medium, extremely low concentrations of TDZ stimulated axillary shoot proliferation of many woody species, including the difficult to propagate walnut tree (*Julgans nigra* L.) (Driver and Kuniyuki, 1984). When designing micropropagation experiments it is often necessary to have a lower range for TDZ than amino purine cytokinins, however the reason for the high level of activity at low concentration of TDZ is not yet understood at the molecular level (Huetteman and Preece, 1993).

Aims

There are many obstacles to overcome when propagating tissues from mature woody plants (see also chapter 1 section 1. 6). The aim of the experimental work in this chapter was to optimise the shoot production phase for a range of genotypes of *F. excelsior*. This was achieved by testing three culture regimes and by optimising the composition of the culture media including nutrient media formulation, carbohydrates and plant growth regulators. The biochemical response of plants in three different culture regimes was tested by analysing the polyamine content of the tissues giving a unique insight of *F. excelsior* shoot cultures *in vitro*.

4. 6. Material and methods

A. Plant material used in experiments

Mature trees of selected genotypes 8, 47, 49, 70, 72 and non-selected genotype F5 were grown as grafted plants in the field and/ or greenhouse at Teagasc, Kinsealy (chapter 3 section 3. 7. A (2)). The unselected seedling plants of genotypes S8 and S11 were initiated from seed obtained from a commercial seed source (Coillte, Ireland). All the culture lines used in experiments were in the *in vitro* culture cycle for at least 18 months. It must be noted that the shoot cultures used in the following experiments were not derived from the initiation experiments described in Chapter 3.

B. Culture media and vessels

Cultures were initiated in 50mm Petri dishes (deep), sealed with Parafilm (one explant per dish) containing 6mls of medium (section 4. 6. C). The standard culture media used for shoot production was M91 medium and QRC medium supplemented with 3g/l activated charcoal (for a full description of media refer to chapter 3 section 3. 7. B). During shoot production, explants were cultured in 200ml powder jars with screw top lids (3 explants per jar) containing 30mls of media. All media was adjusted to pH 5.8 prior to sterilisation in the autoclave at 121°C for 20 minutes. Cultures were maintained in the growth room, at 22°C with 16-hour photoperiod, under Philips cool white fluorescent tubes (75 µmol m^{-2} s^{-1} at bench level).

C. Culture establishment, maintenance and subculture

The cultures from mature trees were initiated by washing apical and nodal explants under running water for one hour followed by sterilisation by immersion in 0.1% mercuric chloride for 15 minutes, washing in sterile distilled water for five minutes, immersion in 7% calcium hypochlorite for 15 minutes and washing three times in sterile distilled water for five minutes each time. Explants were placed on Petri dishes containing M91 medium with double strength TDZ (1.1mg/l). The cultures from seedlings were initiated by sterilising the samaras in 7% calcium hypochlorite for 30 minutes, extracting the embryo and placing it directly on M91 medium with no plant growth regulators. During shoot production, the standard culture regime was to culture shoots for five to six weeks on M91 medium then subculture to QRC media supplemented with activated charcoal at the next

culture period for a further five to six weeks, then subculture shoots back to the M91 medium. In this way, shoots were subcultured onto different medium at every transfer.

During subculturing of the shoots any callus was trimmed from the base, and the shoots were subdivided into nodal explants and shoot tips. The nodal explants consisted of one node and a section of internode below (figure 4. 1. A), or two node explants which had two sets of nodes separated by an internode, the stem was excised above the upper node and about 5mm of internode was left below the lower node. Shoot tip explants consisted of the apical bud and approximately two sets of nodes below (figure 4. 1. B). Explants were approximately 5 to 10mm long. Only vigorously growing shoots were used in the experiments.

Figure 4. 1. A typical single node explant (A) and shoot tip explant (B) used for *in vitro* shoot production experiments.

D. Data collection and analysis

Shoot proliferation experiments lasted for five weeks (unless stated otherwise) under the same cultural conditions as used for stock material. Any contaminated material was discarded from experiments. At the end of the cultivation period recordings were made of viability (due to explant death not contamination), number of new shoots per node, new shoot length, propagation rate (no. of new explants obtained per original live explant cultured), fresh weight and dry weight (DW) (unless stated otherwise). The fresh weight was recorded at the beginning of an experiment by weighing 24 typical explants. To obtain the dry weight of the explants they were placed in paper envelopes and dried in the oven at 100±5 °C for approximately 18 hours. After which the envelopes were removed and the explants weighed immediately. At the end of an experiment the same process was repeated to obtain the fresh and dry weights for each explant. The increase in dry weight of the explants between the beginning and the end of an experiment was expressed as a percentage as follows:

$$\text{Dry weight increase (\%)} = \frac{\text{(end of experiment dry weight - original dry weight)}}{\text{original dry weight}} * 100$$

Statistical analysis was performed using Statistica 6.0 statistical package. Viability data was analysed by the Chi square test (unless stated otherwise), viability data may also have been arc sin transformed to normalise the data, then analysed by ANOVA. Dry weight increase (%) data was log transformed, prior to analysis to normalise the data. Continuous data was analysed by analysis of variance (ANOVA). Mean separation was performed using Tukey's honest significant difference (HSD) test. Significance was recorded at $p \leq 0.05$. All descriptive statistics reported in text and tables is from the non-transformed data, even where the statistical tests were done on the transformed variable.

E. Experimental treatments

Explants for all experiments described below were collected from shoots previously cultured on QRC medium supplemented with 3g/l activated charcoal (unless stated otherwise).

(1) Investigating the effect of three different culturing regimes on shoot production and polyamine concentration.

The explants used in each of the following experiments consisted of a mixture of double nodes and shoot tips, with three explants cultured per jar. Jars were arranged randomly on the same shelf in the growth room. All experiments were started in December 2002. The propagation rate for eight jars per genotype in each experiment was recorded at each five week transfer until the end of the experiment in August 2003. To test the effect of three culturing regimes on the proliferation of ash *in vitro* and on the levels of polyamines produced by the shoots, the three following experiments were set up:

(1. i) The first culture regime involved continuously culturing shoots of genotype F5 and S11 on QRC media supplemented with 3g/l activated charcoal.

(1. ii) The second culture regime involved continuously culturing shoots of genotype F5, S11, 72 and 49 on M91 medium.

(1. iii) The third culture regime involved culturing shoots of genotype F5, S11, 72 and 49 alternatively on M91 medium and the QRC medium with 3g/l activated charcoal (this is the same as the standard culture regime described in section C.).

The primary explants for each experiment were taken from shoots cultured on QRC media with charcoal, leaf samples for polyamine extraction (described in section (1.1) below) were also taken from these shoots and are included as the day 0 reference point in the data set.

(1. 1) Measurement of polyamine levels

Leaf samples, for the extraction and measurement of polyamine levels, were taken at the beginning and end of each 35 day culture period. For the first three culture periods, leaf samples were also taken mid-way through the culture cycle at day 17. Three replicate samples, each consisting of

approximately 200mg of fresh leaf material, were taken for each sampling point. Leaf material was only collected from newly produced leaves not in contact with the medium. Immediately after collection, the leaves were placed into labelled 1.5ml microcentrifuge tubes and frozen in liquid nitrogen. The tubes were stored at -80 °C in the freezer at Teagasc Kinsealy Research Centre, until transported in dry ice to the laboratory at the Centre de Recherche Public Gabriel Lippmann, Luxemborg in September 2003, where they were stored at -70°C until analysis.

Polyamines were extracted from the leaves, this procedure involves treatments that disrupt the plant cell wall and cell membranes to release the cellular constituents into an extraction buffer that contains compounds to protect the polyamines from the activity of endogenous enzymes. The extraction procedures followed the method of Walter and Geuns (1987).

Extraction and dansylation.

120 – 170mg of leaf material frozen in liquid nitrogen was weighed out and placed in a 2ml microcentrifuge tube before being homogenised in 1ml of extraction solution (4% $HClO_4$ containing 5mg/l 1,7-diaminoheptane-2HCl as an internal standard). After 1 hour at 4°C the mixture was centrifuged for 5 minutes at 11 000g at 4°C. 50µl of the supernatant was removed as the extract and placed in a fresh 1.5ml microcentrifuge tube. 100µl of 1M carbonate buffer (pH 9) (82.5g Na_2HCO_3; 35.6g of Na_2CO_3) and 100µl of dansyl chloride solution (7mg Dansyl chloride; 1ml acetone) were added to the extract. After vortexing, a small hole was made in the lid of each tube and the mixture heated for 1 hour at 60°C in the dark to allow evaporation of the acetone. The tubes were then cooled rapidly in ice and 600µl of toluene added. After vigorous shaking the organic phase was recovered in a new 1.5ml microcentrifuge tube, 500µl of hexane was added and the mixture vortexed.

Purification

All purification procedures were performed under a fume hood. 50mg silica gel columns (Chromabound® SiOH, Macherey-Nagel, Düren, Germany) were rinsed with 500µl toluene. Each sample was loaded carefully into a column and rinsed with 300µl toluene, followed by 300µl toluene-triethylamine (10:0.3, v:v). The dansylated polyamines were eluted from the columns with 2 rinses of 400µl ethyl acetate into a new 1.5ml microcentrifuge tube. A small hole was made in the lid of each tube and the elute was dried in a spin vacuum. Samples were stored at this stage in a -80 °C freezer until analysis.

Analysis

To the dried extract, 1ml toluene was added and the tubes placed on a shaker for approximately 5 minutes. The solution was then filtered with a 0.2µm filter into a High Performance liquid Chromotography (HPLC) vial. Polyamines were analysed by HPLC (column Zorbax Bonus-RP 4.6

x 150 mm, Agilent Tech., USA). Dansylated putrescine, diaminoheptane, spermidine and spermine (Sigma, St. Louis, USA) were injected as references.

(2) Investigating the effect of media composition on shoot prodution.

Experiments were set up to test the effect of different components of the culture media including basal salt mixture, carbohydrate source and cytokinin concentration, on shoot proliferation. The individual explants used in each of the experiments consisted of a single node with approximately 5-10mm of stem below. Two individual explants of the same genotype were cultured per jar; there were 24 jars per treatment (12 jars per genotype). The jars in each experiment were arranged randomly on the same shelf in the growth room. The initial fresh and dry weights of 24 typical explants per genotype were recorded prior to experimentation. Recordings were made after five weeks culturing (unless stated otherwise) and included the viability, number of new shoots per node, new shoot length (mm), propagation rate, fresh and dry weight.

(2. 1) Investigating the effect of four culture media (MS, WPM, DKW and OM) on shoot production.

Experiments were set up to test the effects of four basic nutrient media on shoot proliferation. The four media tested were modified MS (containing M91 medium salts), modified WPM, Driver and Kuniyuki Walnut medium (DKW) (Driver and Kuniyuki, 1984) and modified Ruigini Olive Medium (OM) (Ruigini, 1984) with altered concentrations of macro- and micronutrients (see appendix 2). All media contained Sequestrene Fe 330 instead of Fe-EDTA as the source of iron and were supplemented with 5mg/l BAP, 0.55mg/l TDZ and 0.2mg/l IBA, 30g/l sucrose and solidified with 0.9g/l agar. In July 2003 explants of genotype S8 and selected genotype 47 were cultured on all four media and the results recorded. The experiment was repeated in November 2003 using selected genotype 49 to test all four media; there were sufficient explants available of genotype S8 and F5 to test S8 on WPM and DKW and F5 on MS and OM.

(2. 2) Investigating the effect of carbon source on shoot production.

Experiments were set up to test the effect of four sources of carbohydrate on shoot proliferation. M91 media supplemented with the carbohydrates sucrose, mannitol, glucose and a combination of sucrose and mannitol, was tested. The four carbon sources and their concentrations in the culture media, expressed in both molarity (mM) and grams per litre, are listed in table 4. 1. All media had the same concentration of plant growth regulators (BAP, TDZ and IBA) and agar as used in the standard M91 medium.

Table 4. 1. The concentration of four carbon sources tested in M91 medium, to determine the optimum carbohydrate for *in vitro* culture of *F. excelsior.*

Carbon source	*[mM]*	*[g/l]*
Sucrose	87.6	30
Mannitol	87.6	15.95
Glucose	87.6	17.35
Sucrose + mannitol	43.8 + 43.8	15 (sucrose) + 8 (mannitol)

In September 2003 explants of genotype S8 and selected genotype 49 were used to test all four carbohydrate media, recordings were made after six weeks in culture. The experiment was repeated in January 2004 using genotypes S11 and selected genotype 49 to test all four carbohydrate media, recordings were made after seven weeks in culture.

(2. 3) *Investigating the effect of different combinations of the cytokinins BAP and TDZ on shoot production.*

Experiments were set up to test the effect of different combinations of BAP (0, 1 or 5mg/l) and TDZ (0, 0.25, 0.5mg/l) on shoot production using modified OM medium supplemented with 30g/l sucrose and 0.85g/l agar. The concentrations of BAP and TDZ, expressed in mg/l, in the culture media are listed in table 4. 2.

In February 2004 explants of selected genotype 8 and genotype 49 were cultured on all nine media (Table 4. 2. media code A – I) and recordings made after seven weeks culturing.

Table 4. 2. The concentration and combinations of BAP and TDZ used to test the effect of the two cytokinins on shoot proliferation.

Media code	*BAP (mg/l)*	*TDZ (mg/l)*
A	5	0.5
B	5	0.25
C	5	0
D	1	0.5
E	1	0.25
F	1	0
G	0	0.5
H	0	0.25
I	0	0

(3) Investigating the effect of culturing on modified MS medium compared to modified OM medium in two culture regimes, on shoot production.

The standard culture procedure for maintenance of ash cultures at the laboratory in Teagasc, Kinsealy is to use M91 medium containing modified MS salts (alternating with QRC media with activated charcoal, see section 4. 6. C), however experiment (2. 1) in this chapter identified modified OM medium as a superior basal salt mixture to modified MS for culturing ash shoots. Following this experiment, it was decided to test the effect of two culture regimes using the modified MS medium and modified OM medium. Both media were supplemented with BAP (5mg/l), TDZ (0.25mg/l), IBA (2mg/l), sucrose (30g/l) and agar (8.5g/l) (henceforth referred to collectively as modified MS or modified OM media, respectively). The explants used in the following experiments were a mixture of two noded explants and shoot tip explants (cultured three explants per jar). Jars were arranged randomly on the same shelf in the growth room. In November 2003, explants from two genotypes, S11 and selected genotype 49, were cultured on modified MS or modified OM media in two regimes described figuratively in figure 4. 2. In the continuous culture regime, explants were transferred to a fresh jar of the same media (either modified MS or modified OM) at each transfer whereas in the alternating culture treatment explants were transferred to QRC media with activated charcoal every second transfer. Explants were cultured for eight weeks then transferred to fresh media and the propagation rates recorded.

At the second transfer period suitably elongated shoots were taken from genotype 49 in the alternate culture treatment on both modified MS and OM media, and tested for root induction capacity. In total 22 shoots were taken from modified MS media and 15 from modified OM media. Shoots were placed on 6mls of QRC media supplemented with 10mg/l IBA in 50mm Petri dishes (two shoots per dish), sealed with Parafilm and cultured in darkness for six days. The shoots were then transferred to QRC medium with activated charcoal in Petri dishes and cultured in the light. Recordings were made after five weeks.

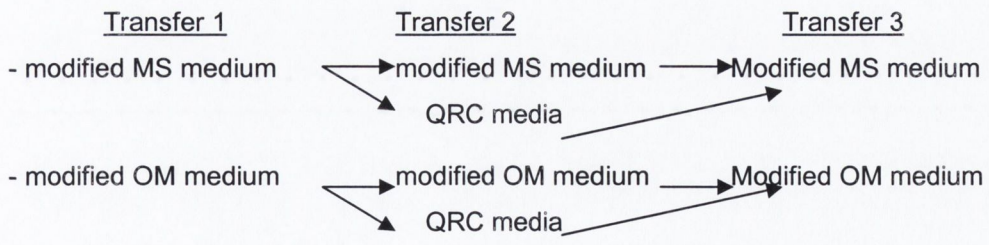


Figure 4. 2. Flow diagram showing the two culture regimes set up to test the effect of culturing on modified MS medium compared to modified OM medium (the arrow signifies a transfer of cultures).

(4) *Investigating the effect of culturing on modified MS and modified OM type A compared to modified MS and modified OM type B on shoot production and health.*

At the end of the shoot production experiments it was discovered that the modified MS and the modified OM medium salts being used throughout, contained micronutrients at a concentration of 10 fold higher than that given in the original publications (Murashige and Skoog, 1962; Ruigini, 1984); furthermore in the modified OM medium, the concentrations of $CaCl_2.2H_20$, $Ca(NO_3)_2.4H_2O$ and $MgSO_4.7H_2O$ were lower than that of the original Ruigini olive medium. Hence, it was necessary to conduct an experiment to compare the modified MS and modified OM media salts used for the previous experiments (hereby referred to as modified MS and OM type B) to media containing the proper intended nutrient levels (hereby referred to as modified MS and OM type A), a full comparison of the nutrients in both media is contained in the appendix 3 and 4. All media were supplemented with BAP (5mg/l), TDZ (0.25mg/l), IBA (2mg/l), sucrose (30g/l) and solidified with agar (8.5g/l). Single node explants of genotype 54 (obtained from Lars Sommer, Vitroform, Denmark) were cultured on modified MS media type A and B and also on modified OM type A and B. Single node explants of selected genotype 49 were cultured on modified OM type A and B. There were 12 jars (containing two individuals per jar) per genotype for each media tested. All jars were arranged randomly on the same shelf in the growth room. Recordings were made after seven weeks culturing.

4. 7. Results

(1) Description of shoot development in the standard culture regime used for the maintenance of ash shoot cultures in vitro at the laboratory at Teagasc Kinsealy Research Centre, Dublin.

The standard culture regime of transferring shoots from M91 media to QRC media with charcoal and *visa versa* was described in section 4. 6. C. The buds on the shoot tips or nodal explants would begin to grow approximately two weeks after they were subcultured. Generally the nodal explants developed more rapidly than the shoot tip explants which in some cases would not grow until the second transfer after excision. Figure 4. 3 shows the typical appearance of shoots on both the M91 media and QRC media with activated charcoal. On the culture media with cytokinins (M91 medium) the explants would generally form a cluster of short shoots and callus with very few shoots over 10mm in length being produced. The explants on QRC media with charcoal were generally elongated with little axillary branching, spontaneous rooting of shoots was frequently observed on this medium. In some cases, endogenous contaminants (suspected to be bacteria) emerged from the cultures, on either media, and reduced the vigour of the shoot.

Figure 4. 3. Typical culture jars containing explants of *F. excelsior*. The jar on the left hand side contains M91 media and the jar on the right hand side contains QRC media with charcoal.

(2) The effects of three different culturing regimes on shoot production and polyamine concentration.

Experiments involving genotypes S11, F5, 72 and 49 were conducted to test the effect of three culturing regimes on the proliferation of ash *in vitro* and on the levels of polyamines produced by the shoots (section 4. 6. E. (1)). The results for each genotype in each culture regime are described in the following pages. Tables of the mean putrescine, spermidine and spermine (nmol g^{-1} FW) content measured at each sampling point are located in appendix 5.

(2.1) Genotype S11

The propagation rates for genotype S11 at each transfer in the three culturing regimes are shown in table 4. 3. The propagation rate in culture regime 1 decreased significantly from day 0 to the transfer at day 35 (One-way ANOVA, $p<0.0001$). The cultures in this regime had become badly contaminated with bacteria and before the next transfer, due at day 70, all were removed from the experiment. In culture regime 2 the propagation rate decreased significantly from day 0 to day 175 (Tukey's HSD, $p<0.00002$) and it was noted on day 52 that the explants had become vitrified, had small spindly leaves and were contaminated with bacteria (see figure 4. 4). The propagation rate decreased significantly in culture regime 3 from day 0 to day 245 (Tukey's HSD, $p<0.0003$). Contamination with bacteria was most apparent when explants were cultured on M91 medium, and the propagation rates were lowest for material grown on this medium compared to those grown on QRC media with charcoal. There was no significant difference between culture regimes for the mean propagation rate recorded over all the transfers (One-way ANOVA, $p=0.5$).

Table 4. 3. The effect of three culture regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) on the propagation rate of shoots of genotype S11.

Days in culture	*Culture regime*			
	1 (QRC – QRC)	*2 (M91 – M91)*	*3 (QRC – M91)*	
0	2.2	2.2	2.2	QRC**
35 (1)*	1.2	1.4	1.4	M91
70 (2)		2.2	2.2	QRC
105 (3)		1.8	1.5	M91
140 (4)		1.2	1.2	QRC
175 (5)		1.3	0.9	M91
210 (6)			2.2	QRC
245 (7)			1.2	M91
Mean ± S. E***	1.7 ± 0.09	1.7 ± 0.05	1.6 ± 0.04	

* Values in parentheses refer to the subculture (transfer) number, i.e. day 35 was the first subculture onto fresh medium since the beginning of the experiment at day 0. **Refers to the media in the alternate culture regime that the shoots were growing on prior to subculture i.e. day 35 shoots subcultured off M91 medium gave a propagation rate of 1.4. *** ± Standard error of the mean.

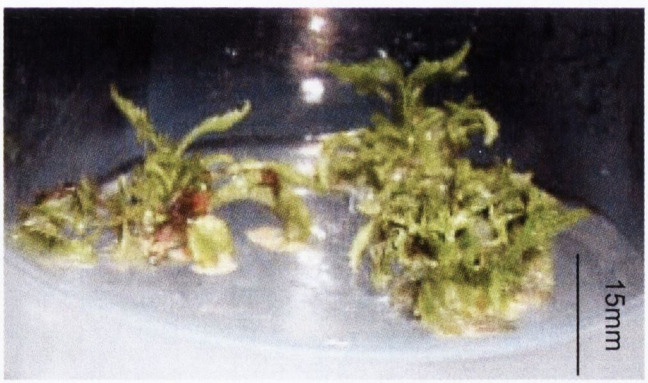


Figure 4. 4. Explants of genotype S11 in culture regime 2 suffering from vitrification, spindly leaves and bacterial contamination.

Polyamines were extracted and measured from leaf samples collected at the beginning and end of each 35 day culture period. For the first three culture periods, samples were also analysed mid-way through the culture cycle at day 17. In culture regime 1 there was no change in the level of putrescine in the leaves measured over time for genotype S11 (One-way ANOVA, $p=0.24$, figure 4. 5). In culture regime 2 the concentration of putrescine increased significantly from the beginning to the end of the experiment (11.1 $nmol.g^{-1}$ FW at day 0 to 1134.7 $nmol.g^{-1}$ FW at day 175) (Tukey's HSD, $p<0.000$). The influence of each transfer on to fresh M91 medium could be seen between day 35 and day 105 where the level of putrescine increased at the measurement 17 days after

each transfer (day 52 and day 87), then decreased at the end of each transfer period (day 70 and day 105). In culture regime 3, where the shoots were transferred to M91 medium or QRC medium with charcoal at each alternative culture period, the putrescine concentration increased significantly from day 0 to day 210 (Tukey's HSD, $p<0.003$). The influence of transferring the shoots to the different culture media can be seen between day 105 and 210 in culture regime 3, leaves taken from shoots on M91 media (day 105 and 175) had higher levels of putrescine compared to those taken off QRC medium with activated charcoal (day 140 and 210), however these differences were not significant according to Tukey's HSD.

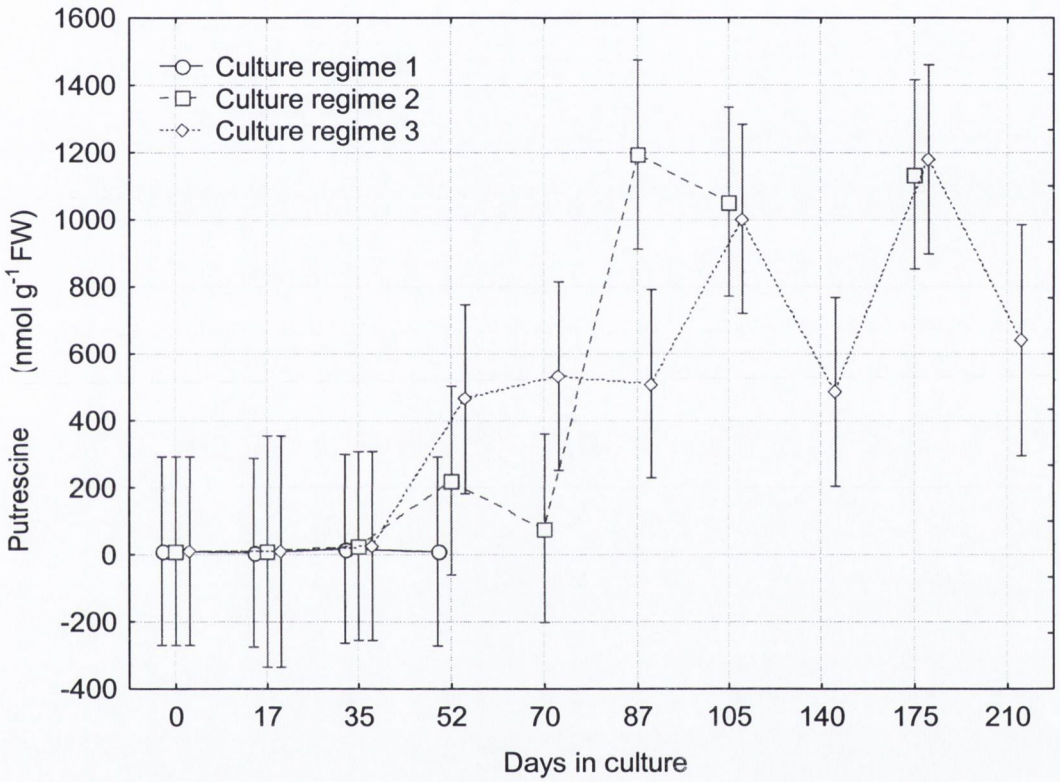


Figure 4. 5. The concentration of putrescine (nmol g^{-1} FW) in leaf samples from shoots of genotype S11 cultured in three culturing regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

The results in table 4. 4. show the mean concentration of each polyamine in each culture regime, for genotype S11, measured over the duration of the experiment. The mean concentration of putrescine varied significantly between culture regimes 1, 2 and 3 (One-way ANOVA, $p<0.00005$). The level of putrescine in plants of genotype S11 from culture regime 1 was significantly lower compared to the other two culture regimes (Tukey's HSD, culture regime 2 $P<0.003$ and culture regime 3 $p<0.0006$).

Table 4. 4. The effect of culture regime on the mean concentration of putrescine, spermidine and spermine in leaf samples of genotypes S11, F5, 72 and 49 over a total of 245 days.

Genotype	Polyamine								
	Putrescine (nmol g^{-1} FW ± s. e.*)			Spermidine (nmol g^{-1} FW ± s. e.*)			Spermine (nmol g^{-1} FW ± s. e.*)		
	Culture regime			Culture regime			Culture regime		
	1	2	3	1	2	3	1	2	3
S11	11.2 ± 1.8^{Aa}**	485.9 ± 119.5^{Ab}	497.7 ±84.6^{Ab}	13.4 ±2.2^{Aa}	87.9±18.7^{Ab}	57.9±11.3^{Aab}	8.2±1.7^{Aa}	33.7±8.4^{Aa}	11.9±2.4^{Aa}
F5	7.4 ± 0.8^{Aa}	553.5 ± 131.9^{Ab}	407.6±89.6^{Ab}	13.9±1.5^{Aa}	395.7±53.4^{Bb}	129.4±19.6^{Ac}	3.9±0.5^{Ba}	166.9±30.6^{Bb}	74.2±18.2^{Bb}
72		885.6±251.6^{Aa}	856.8±263.9^{Aa}		221.9±37.9^{ABa}	109.9±19.5^{Aa}		92.6±17.8^{ABa}	54.7±13.4^{ABa}
49		577.9±147.7^{Aa}	524.1±184.9^{Aa}		215.4±39.7^{Aa}	124.8±34.6^{Aa}		95.4±13.7^{ABa}	40.6±10.1^{ABb}
Mean	8.6 ± 0.9	622.4 ± 85.4	*578.3 ± 88.7*	13.3 ± 1.1	229.1 ±22.2	102.9 ± 11.4	5.1 ± 0.7	98.1 ± 10.7	*45.3 ± 6.4*

* ± Standard error. ** Different letters between rows (uppercase) and columns (lowercase) within each polyamine section and each genotype indicate a significant difference according to Tukeys unequal N HSD at the 5% level. For example, in experiment 1 the putrescine level in genotype S11 is not significantly different to that of genotype F5 (uppercase letters), however the putrescine level in genotype S11 in experiment 1 is significantly different to that in experiment 2 and 3 (lowercase letters).

There was no significant variation in the level of spermidine measured over time in shoots cultured in regime 1 (One-way ANOVA, p=0.1, figure 4. 6). In culture regime 2 the level of spermidine measured in the cultures increased significantly from day 0 (7.0 nmol g^{-1} FW) to day 175 (206.3 nmol g^{-1} FW) (Tukey's HSD, p<0.0001). There was no significant increase in spermidine concentration between the beginning and end of culture regime 3 (Tukey's HSD, p=0.6). However, the influence of transferring cultures from M91 media to QRC media with charcoal could be seen from day 105 – 210 (culture regime 3). The level of spermidine in leaves taken from cultures on M91 media on day 105 and 175 is higher than that taken from those on QRC media with charcoal on day 140 and 210. In addition, the spermidine content in leaves taken from shoots on M91 media at day 105 and 175 was significantly higher than that in leaves at day 0 (Tukey's HSD, p<0.002, for both comparisons).

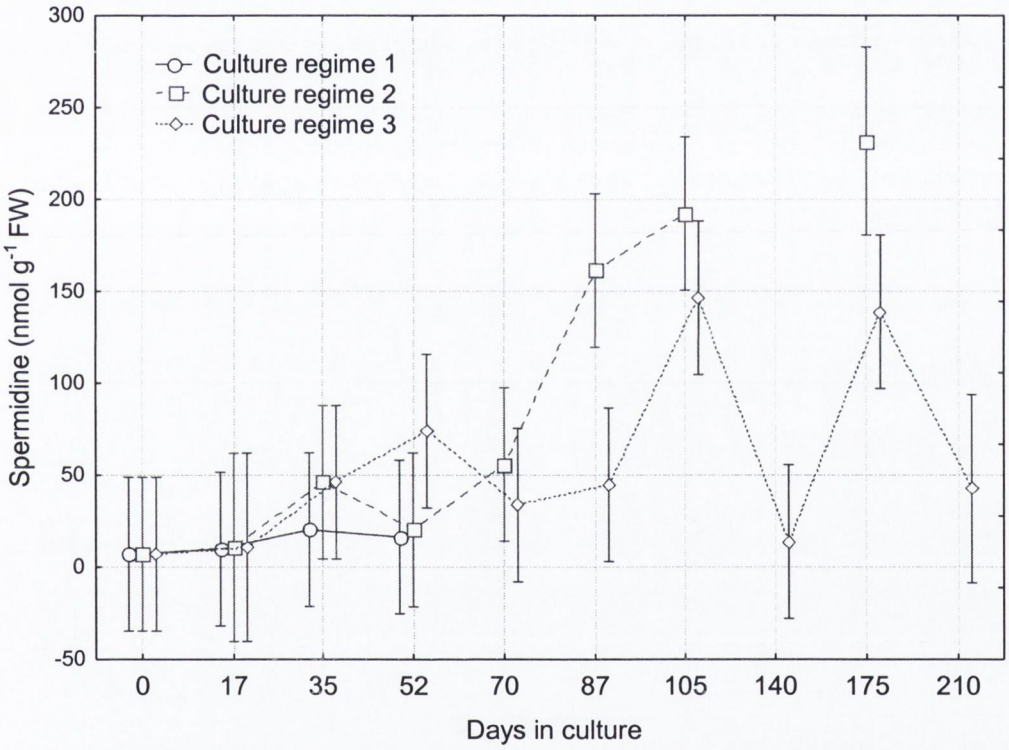


Figure 4. 6. The concentration of spermidine (nmol g^{-1} FW) in leaf samples from shoots of genotype S11 cultured in three culturing regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

The results in table 4. 4. show that the mean concentration of spermidine varied significantly between culture regimes 1, 2 and 3 (One-way ANOVA, p<0.003). The level of spermidine in culture regime 2 was significantly higher than that in culture regime 1, but similar to that of culture regime 3 (Tukey's HSD, culture regime 1 p<0.009).

There was no significant variation in the level of spermine measured over time in culture regime 1 (One-way ANOVA, p=0.2, figure 4. 7). In culture regime 2 the level of spermine measured in the cultures increased significantly from day 0 (4.7 nmol.g^{-1} FW) to day 175 (94.4 nmol.g^{-1} FW) (Tukey's HSD, p<0.02). There was no significant increase in spermine concentration between the beginning and end of culture regime 3 (Tukey's HSD, p=0.9). However, the influence of transferring cultures from M91 media to QRC media with charcoal could be seen from day 105 – 210. The level of spermine in leaves taken from cultures on M91 media is higher than those on QRC media with charcoal. The spermine content in leaves taken from shoots on M91 media at day 175 was significantly higher than that in leaves at day 0 (Tukey's HSD, p<0.003).

The results in table 4. 4 show that the mean concentration of spermine did not vary significantly between culture regimes (One-way ANOVA, p=0.2), however the highest level was recorded in culture regime 2. For all three polyamines the lowest levels recorded were in culture regime 1.

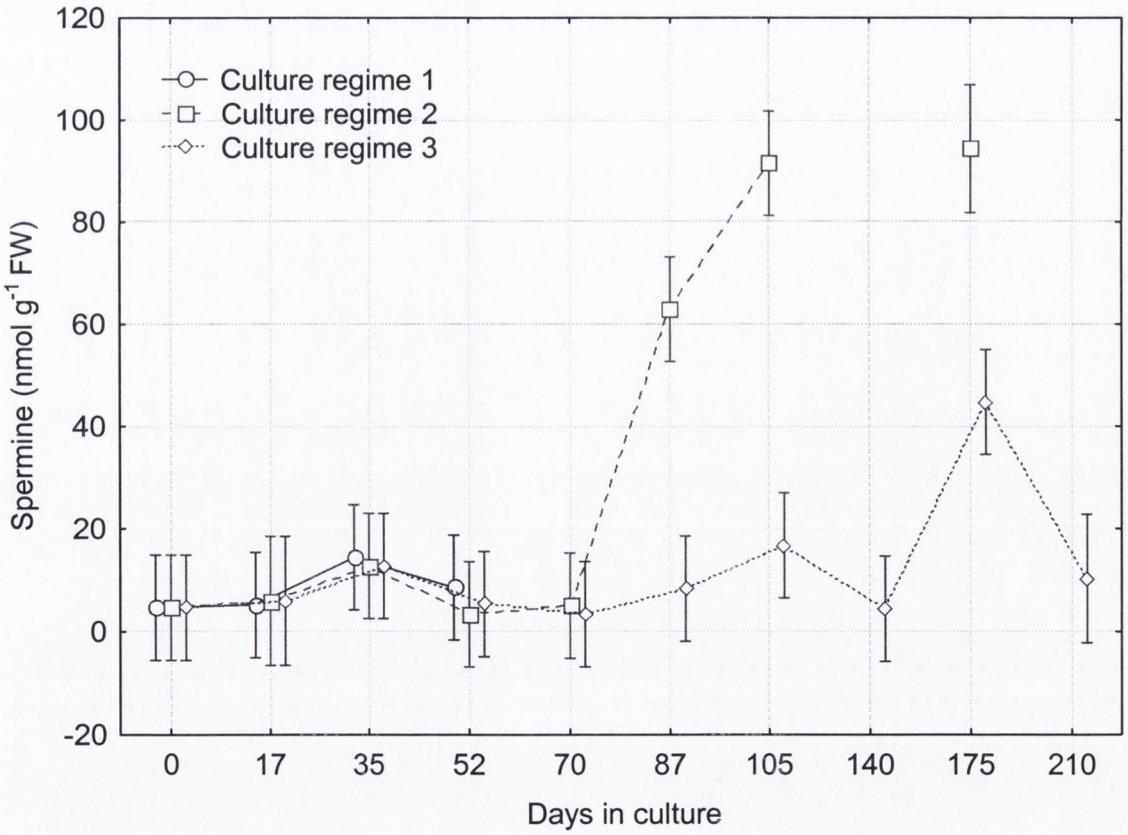


Figure 4. 7. The concentration of spermine (nmol g^{-1} FW) in leaf samples from shoots of genotype S11 cultured in three culturing regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

(2. 2) Genotype F5

The propagation rates for genotype F5 at each transfer in the three culture regimes are shown in table 4. 5. The propagation rate in culture regime 1 increased significantly from day 0 to day 70 (Tukey's HSD, $p<0.04$), then decreased steadily with each subsequent transfer. In culture regime 2 the propagation rate increased significantly after the first two transfers on M91 medium (day 35 and 70) compared to day 0 (Tukey's HSD, $p<0.0003$, for both comparisons) and it was noted that the shoots were growing well. The propagation rate then decreased after day 70 and the cultures started to show bacterial contamination. The propagation rate varied significantly with each transfer in culture regime 3 (One-way ANOVA, $p<0.000$); rates increased significantly from day 0 to day 70 (Tukey's HSD, $p<0.00003$) then decreased after day 105 mainly due to a build up of bacteria. Presence of bacteria was most apparent when shoots were cultured on the M91 medium. The mean propagation rate recorded overall the transfers was significantly higher in culture regime 3 compared to the other two culture regimes (Tukey's HSD, culture regime 1, $p<0.00005$; and culture regime 3 $p<0.00003$).

Table 4. 5. The effect of three culture regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) on the propagation rate of shoots of genotype F5.

Days in culture	*Culture regime*			
	1 (QRC – QRC)	*2 (M91 – M91)*	*3 (QRC – M91)*	
0	1.6	1.6	1.6	QRC**
35 (1)*	1.5	2.2	2.2	M91
70 (2)	2.0	2.2	2.5	QRC
105 (3)	1.7	1.4	1.6	M91
140 (4)	1.5	0.8	1.5	QRC
175 (5)	1.3	1.3	1.7	M91
210 (6)			3.0	QRC
245 (7)			1.3	M91
Mean ± S. E. ***	1.6 ± 0.04	1.6 ± 0.05	1.9 ± 0.05	

* Values in parentheses refer to the number of subcultures, i.e. day 35 was the first subculture onto fresh medium since the beginning of the experiment at day 0. **Refers to the media in the alternate culture regime that the shoots were growing on prior to subculture i.e. day 35 shoots subcultured off M91 medium gave a propagation rate of 2.2. ***± Standard error of the mean.

Polyamine levels were measured in each culture regime. In culture regime 1 there was no significant change in the level of putrescine measured over time (One-way ANOVA, $p=0.37$, figure 4. 8). In culture regime 2 the concentration of putrescine increased steadily and significantly from 7.1 nmol.g^{-1} FW at day 0 to 1661.5 nmol.g^{-1} FW at day 175 (Tukey's HSD, $p<0.000$). The

putrescine concentration at day 105 in culture regime 2 appears unusually low; this may have be due to defrosting of that set of samples which occurred in February and March 2003. In culture regime 3 the concentration of putrescine increased significantly from day 0 to day 245 (Tukey's HSD, $p<0.0003$), and an increased concentration of putrescine from shoots on M91 media (days 175 and 245) compared to QRC media with charcoal (day 210) can be seen towards the end of the experiment.

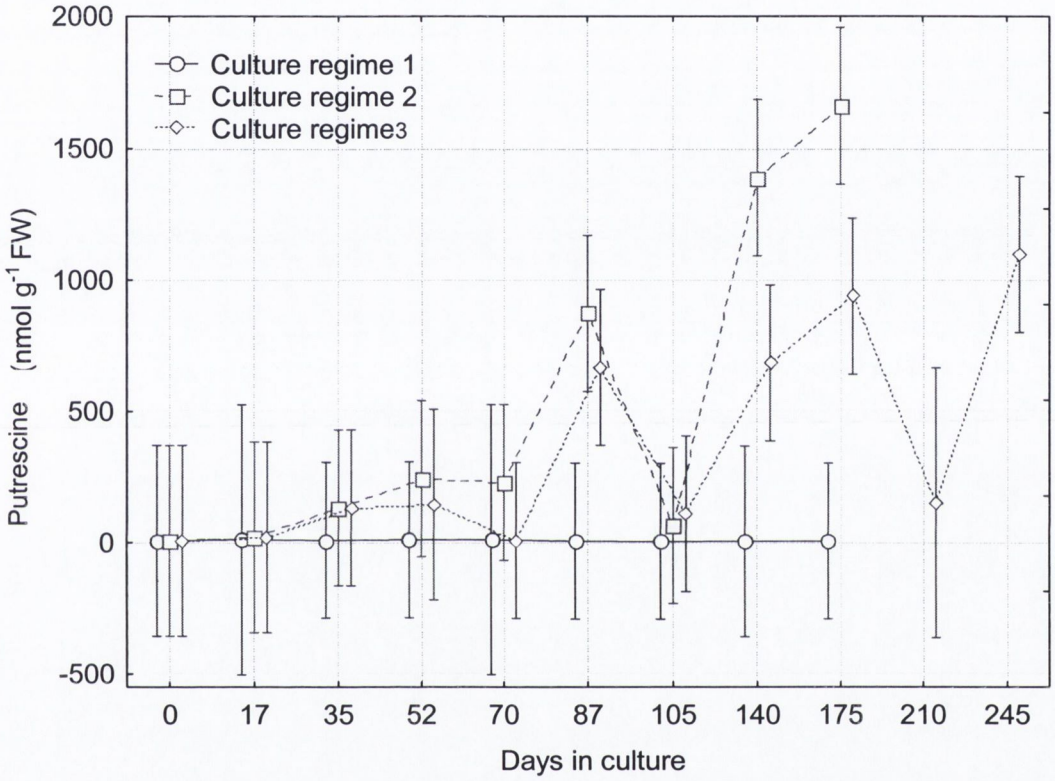


Figure 4. 8. The concentration of putrescine (nmol g^{-1} FW) in leaf samples from shoots of genotype F5 cultured in three culturing regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

The results in table 4. 4 show that the mean concentration of putrescine varied significantly between culture regimes 1, 2 and 3 (One-way ANOVA, $p<0.000$) and was significantly higher in culture regime 2 compared to the other two culture regimes (Tukey's HSD, culture regime 1 $p<0.0007$ and culture regime 3 $p<0.0001$).

In culture regime 1, the concentration of spermidine was low from day 0, and there was no significant change concentration from the beginning to the end of the culture regime (day 0 - day

175) (Tukey's HSD, p=0.06, figure 4. 9). The concentration of spermidine measured from the leaves of shoots in culture regime 2 increased significantly from 17.0 nmol.g^{-1} FW at day 0 to 648.9 nmol.g^{-1} FW at day 105 (Tukey's HSD, p<0.002). After day 105 the content of spermidine began to decline in the leaves, and at day 175 was measured at 215.8 nmol.g^{-1} FW. In culture regime 3, the concentration of spermidine fluctuated with each transfer, increasing after the shoots are transferred to M91 media and decreasing after transfer to QRC media with charcoal especially after day 52 (eg. spermidine concentration increased from 44.0 to 297.9 nmol.g^{-1} FW on M91 media from day 70 to 105 then decreased to 48.7 nmol.g^{-1} FW on day 140 after 35 days on QRC media with charcoal).

The results in table 4. 4 show that the mean concentration of spermidine varied significantly between culture regimes 1, 2 and 3 (One-way ANOVA, p<0.0001) and was significantly higher in culture regime 2 compared to the other two culture regimes (Tukey's HSD, culture regime 1 P<0.0001 and culture regime 3 p<0.004). The level of spermidine measured in culture regime 1 was significantly lower than that in culture regime 3 (Tukey's HSD, P<0.0001).

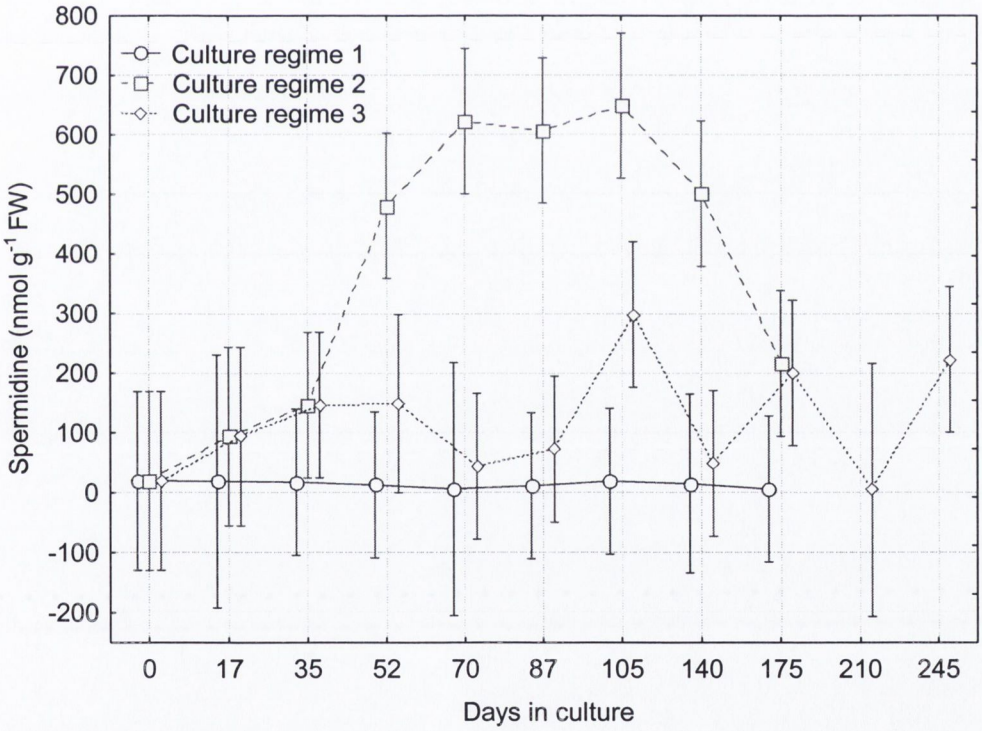


Figure 4. 9. The concentration of spermidine (nmol g^{-1} FW) in leaf samples from shoots of genotype F5 cultured in three culturing regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

In culture regime 1 there was no significant change in the concentration of spermine measured at day 0 and day 175 (Tukey's HSD, p=0.14, figure 4. 10). In culture regime 2 the concentration of spermine increased steadily until day 140 (427.9 nmol g^{-1} FW), then decreased after the last transfer on day 175 (120.2 nmol g^{-1} FW). However, the concentration of spermine on day 175 was still significantly higher to that at the beginning of the experiment on day 0 (2.5 nmol g^{-1} FW) (Tukey's HSD, p<0.002). In culture regime 3, the concentration of spermine fluctuated with each transfer, increasing after the shoots were transferred to M91 media and decreasing after they were transferred to QRC media with charcoal eg. spermine concentration increased from 11.1 to 175.0 nmol g^{-1} FW on M91 media from day 70 to 105 then decreased to 22.7 nmol g^{-1} FW on day 140 after 35 days on QRC media with charcoal.

The results in table 4. 4 show that the mean concentration of spermine varied significantly between the culture regimes (One-way ANOVA, p<0.000) and was lowest in culture regime 1 compared to the other two regimes (Tukey's HSD, culture regime 2 P<0.0001 and culture regime 3 p<0.0001). The highest level of spermine was recorded in culture regime 2. For all three polyamines the lowest levels were recorded in culture regime 1 and the highest levels were recorded in culture regime 2.

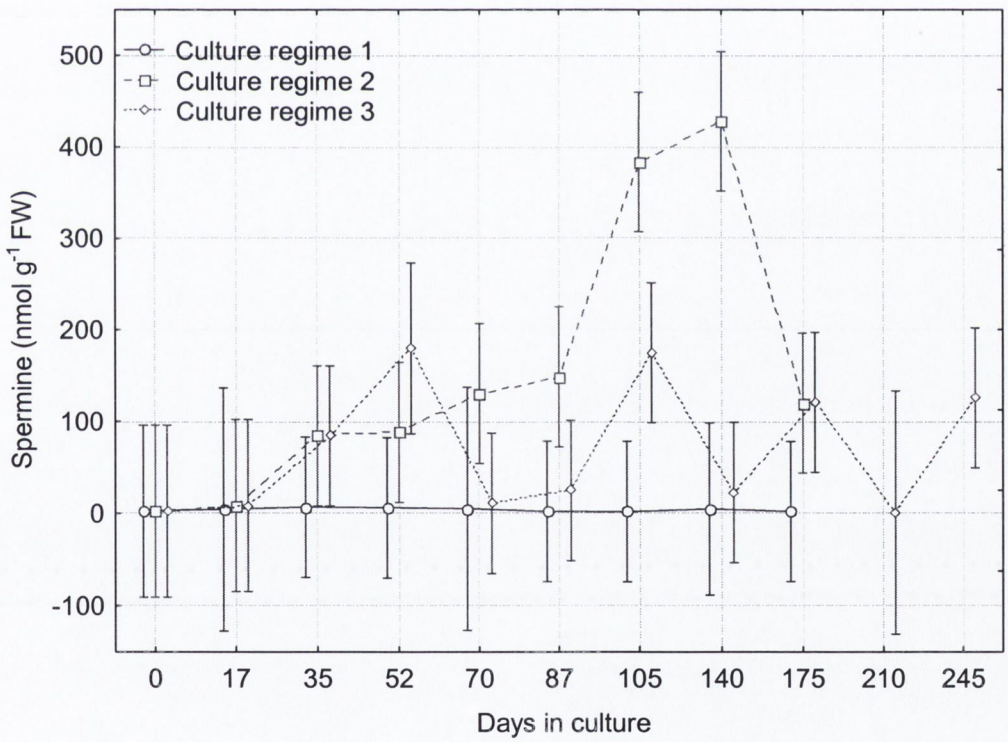


Figure 4. 10. The concentration of spermine (nmol g^{-1} FW) in leaf samples from shoots of genotype F5 cultured in three culturing regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

(2.3) Genotype 72

The propagation rates for genotype 72 at each transfer in culture regimes 2 and 3 are shown in table 4. 6. In culture regime 2 the propagation rate increased significantly from day 0 to day 70 (Tukey's HSD, $p<0.0002$), the propagation rate decreased after day 70 and it was noted on day 105 that approximately 24% of shoots were necrotic and the remainder were suffering from vitrification, excess callus production and bacterial contamination (figure 4. 11). In culture regime 3, the propagation rates from shoots on QRC media with charcoal were generally higher than those from shoots on M91 media. Bacterial contamination was apparent in the cultures after day 105 and was particularly problematic when shoots were transferred to M91 medium. The mean propagation rate recorded over all the transfers was significantly higher in culture regime 3 compared to culture regime 2 (Tukey's HSD, $p<0.04$).

Table 4. 6. The effect of two culture regimes (1: continuous culture on QRC with charcoal; 2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) on the propagation rate of shoots of genotype 72.

Days in culture	*Culture regime*		
	2 (M91 – M91)	*3 (QRC – M91)*	
0	1.3	1.3	QRC**
35 (1)*	2.3	2.3	M91
70 (2)	2.3	2.1	QRC
105 (3)	1.0	1.4	M91
140 (4)	1.1	1.7	QRC
175 (5)	1.7	1.9	M91
210 (6)		2.4	QRC
245 (7)		1.2	M91
Mean ± S. E. ***	1.6 ± 0.07	1.8 ± 0.04	

* Values in parentheses refer to the number of subcultures, i.e. day 35 was the first subculture onto fresh medium since the beginning of the experiment at day 0. **Refers to the media in the alternate culture regime that the shoots were growing on prior to subculture i.e. day 35 shoots subcultured off M91 medium gave a propagation rate of 2.3. ***± Standard error of the mean.

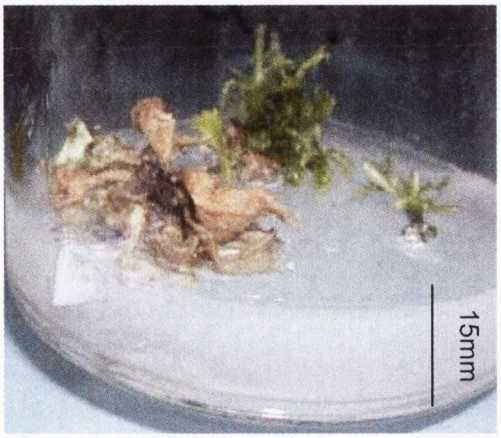


Figure 4. 11. Explants of genotype 72 in culture regime 2 suffering from necrosis and spindly leaves.

In culture regime 2 the concentration of putrescine increased significantly from day 0 to day 175 (Tukey's HSD, $p<0.00007$, figure 4. 12). The concentration of putrescine measured at the end of this culture regime was among the highest over all the culture regimes and genotypes. The concentration of putrescine in culture regime 2 increased steadily from 66.6 nmol g^{-1} FW at day 0 to 815.5 nmol.g^{-1} FW at day 52. On day 35 it was noted that the explants were producing large amounts of callus and on day 52 they had become contaminated with bacteria and the leaves were vitrified. At day 70 the putrescine concentration decreased to 161.7 nmol g^{-1} FW and continued to decline until after day 105. It was noted that on day 105 approximately 24% of shoots had died and the remainder were vitrified and/ or contaminated with bacteria. At the next sampling period on day 140 the putrescine concentration had risen dramatically to 2756.1 nmol g^{-1} FW. In culture regime 3, it is possible to see the effect of each transfer on the concentration of putrescine in the leaves. Generally there was an increase in putrescine content after the explants were transferred to M91 medium followed by a decrease when transferred to QRC medium with charcoal. The highest concentration of putrescine was measured on day 105 when it was noted that 70% of explants were contaminated with bacteria and 36% were necrotic. The concentration of putrescine in the leaves increased significantly from the beginning to the end of the culture regime (Tukey's HSD, $p<0.01$).

The results in table 4. 4 show that the mean concentration of putrescine was similar between culture regime 2 and 3 (One-way ANOVA, $p=0.55$).

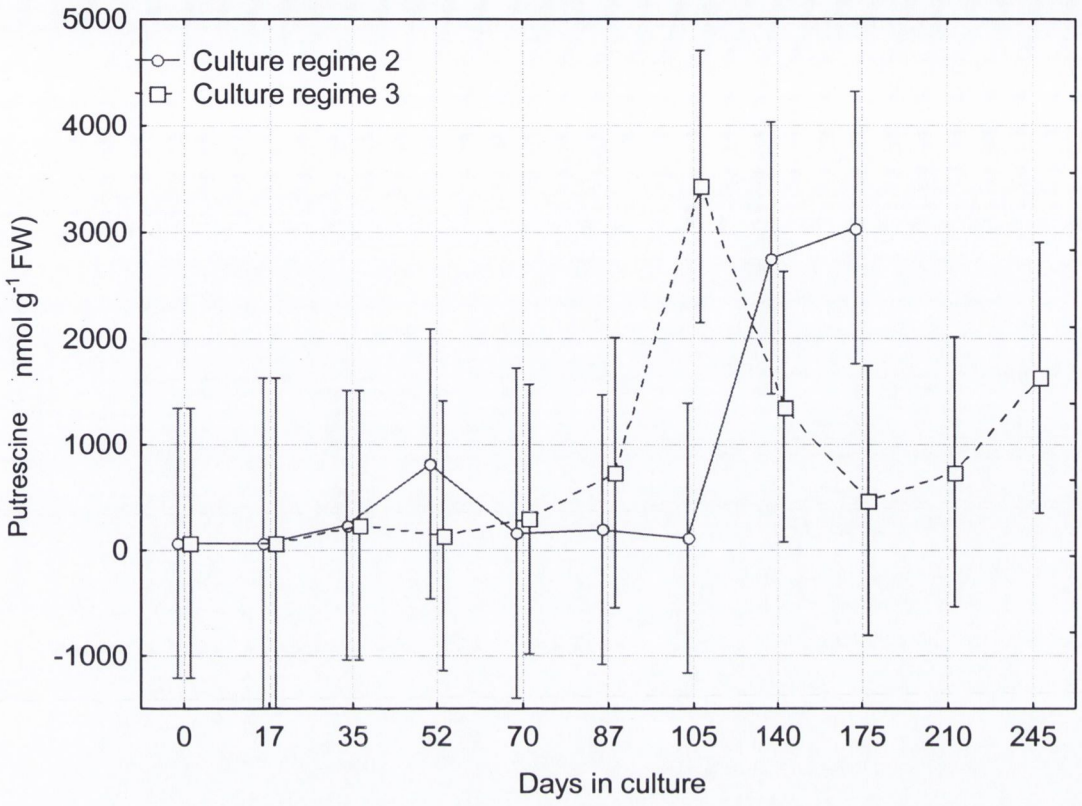


Figure 4. 12. The concentration of putrescine (nmol g^{-1} FW) in leaf samples from shoots of genotype 72 cultured in two culturing regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

The concentration of spermidine increased sporadically during culture regime 2 and was significantly higher at the end of the culture regime compared to the beginning (Tukey's HSD, $p<0.0002$, figure 4. 13). In culture regime 3, the transfer of shoots between the two media was noticeable by the change in spermidine concentration which increased after transfer to M91 media and generally decreased when shoots were cultured on QRC with charcoal. The concentration of spermidine at the end of the experiment was significantly higher than that at the beginning (Tukey's HSD, $p<0.01$).

The results in table 4. 4 show that the mean concentration of spermidine was similar between culture regime 2 and 3 (One-way ANOVA, $p=0.07$).

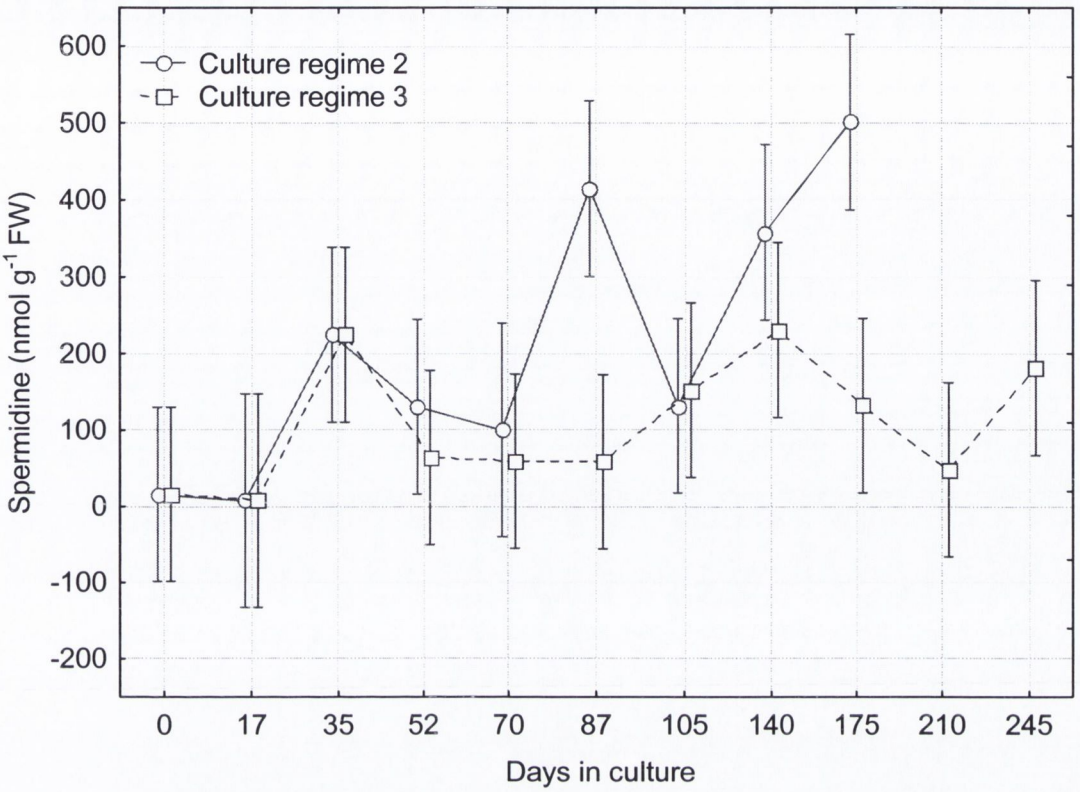


Figure 4. 13. The concentration of spermidine (nmol g^{-1} FW) in leaf samples from shoots of genotype 72 cultured in two culturing regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

The concentration of spermine in culture regime 2 increased up to day 35 then began to drop until day 87 when an increase was recorded which continued until the penultimate sampling date (figure 4. 14). A similar drop in polyamine level was recorded for spermidine between days 35 and 70 and for putrescine slightly later between days 52 and 140. In culture regime 3, there was no significant difference between spermine concentration recorded at the beginning and the end of the experiment (Tukey's HSD, $p=0.75$). However, the concentration had increased and was generally higher on M91 medium compared to QRC medium with charcoal. Except on day 140, where the highest concentration of spermine was found in leaves from shoots cultured on QRC medium with charcoal, this was significantly higher than the concentration of spermine on day 0 and day 17 only (Tukey's HSD, $p<0.01$ for both comparisons).

The results in table 4. 4 show that the mean concentration of spermine was similar between culture regime 2 and 3 (One-way ANOVA, $p=0.09$). For all three polyamines, the highest levels were recorded in culture regime 2.

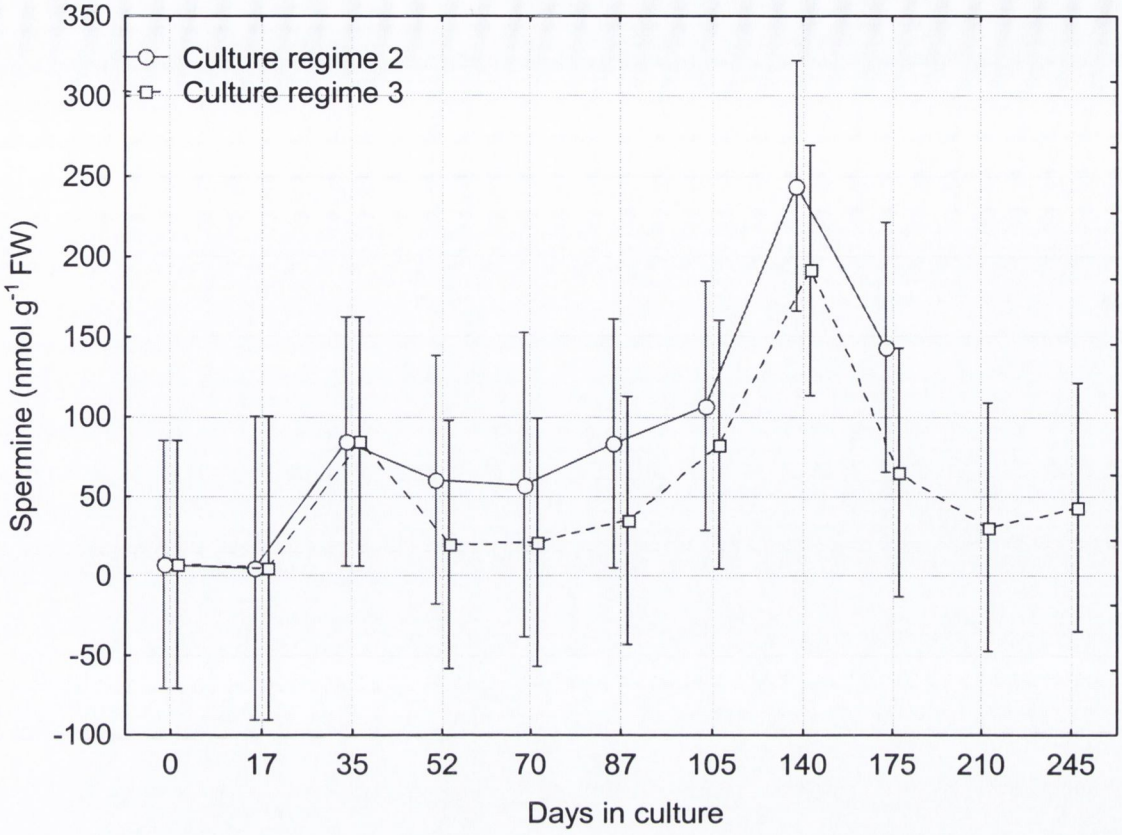


Figure 4. 14. The concentration of spermine (nmol g^{-1} FW) in leaf samples from shoots of genotype 72 cultured in two culturing regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

(2. 4) Genotype 49

The propagation rates for genotype 49 at each transfer in culture regimes 2 and 3 are shown in table 4. 7. In culture regime 2 there was a significant decrease in propagation rate over time from day 0 to day 175 (Tukey's HSD, $p<0.00002$). A similar result was obtained for culture regime 3, the propagation rate at the end of the experiment was significantly lower compared to that at the beginning (Tukey's HSD, $p<0.00003$). The propagation rates from shoots cultured on QRC medium with charcoal were higher than those from M91 medium. The mean propagation rate recorded overall the transfers was not significantly different between culture regimes 2 and 3 (One-way ANOVA, $p=0.06$).

Table 4. 7. The effect of two culture regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) on the propagation rate of shoots of genotype 49.

Days in culture	*Culture regime*		
	2 (M91 – M91)	*3 (QRC – M91)*	
0	3.5	3.5	QRC**
35 (1)*	2.2	2.2	M91
70 (2)	2.5	2.6	QRC
105 (3)	1.8	1.8	M91
140 (4)	2.4	2.1	QRC
175 (5)	2.1	1.5	M91
210 (6)		3.0	QRC
245 (7)		1.4	M91
Mean ± S. E.***	2.4 ± 0.06	2.3 ± 0.06	

* Values in parentheses refer to the number of subcultures, i.e. day 35 was the first subculture onto fresh medium since the beginning of the experiment at day 0. **Refers to the media in the alternate culture regime that the shoots were growing on prior to subculture i.e. day 35 shoots subcultured off M91 medium gave a propagation rate of 2.2. ***± Standard error of the mean.

In culture regime 2 the concentration of putrescine increased significantly from day 0 to day 175 (Tukey's HSD, $p<0.005$, figure 4. 15). During the culture regime the concentration fluctuated after each transfer, originally decreasing on day 17 after the initial transfer to M91 medium. The trend for each subsequent transfer was for the putrescine concentration to increase after transfer to fresh medium, then decrease at the end of the transfer period. In culture regime 3, the putrescine concentration at the end of the experiment was not significantly higher than at the beginning (Tukey's HSD, $p=0.47$). After day 52, the concentration of putrescine measured from the leaves tended to increase after transfer to fresh medium, then decrease at the end of the transfer period, in a similar pattern to that measured for culture regime 2. After day 140 the putrescine concentration decreased with each transfer and at the end of the experiment was the lowest compared to any other genotype in culture regime 3 (day 245: 30.3 nmol g^{-1} FW). The results in table 4. 4 show that the mean concentration of putrescine was similar between culture regime 2 and 3 (One-way ANOVA, $p=0.96$).

The spermidine concentration in culture regime 2 increased during the experiment and at the end was significantly higher than on day 0 (Tukey's HSD, $p<0.0002$, figure 4. 16). The spermidine levels measured in the leaves fluctuated with each transfer, increasing initially on day 52 (after the second transfer to fresh M91 medium) then decreasing at the end of the culture cycle and increasing again during the third transfer to fresh medium (day 87). This trend continued until the end of the experiment. In culture regime 3, the spermidine concentration at the beginning of the

experiment was similar to that at the end (Tukey's HSD, p=0.99). The only significant increase in spermidine concentration, when compared to day 0, was recorded on days 70, 87 and 175 (Tukey's HSD, p<0.003, p<0.02 and p<0.02, respectively). The results in table 4. 4 show that the mean concentration of spermidine levels was similar between culture regime 2 and 3 (One-way ANOVA, p=0.09).

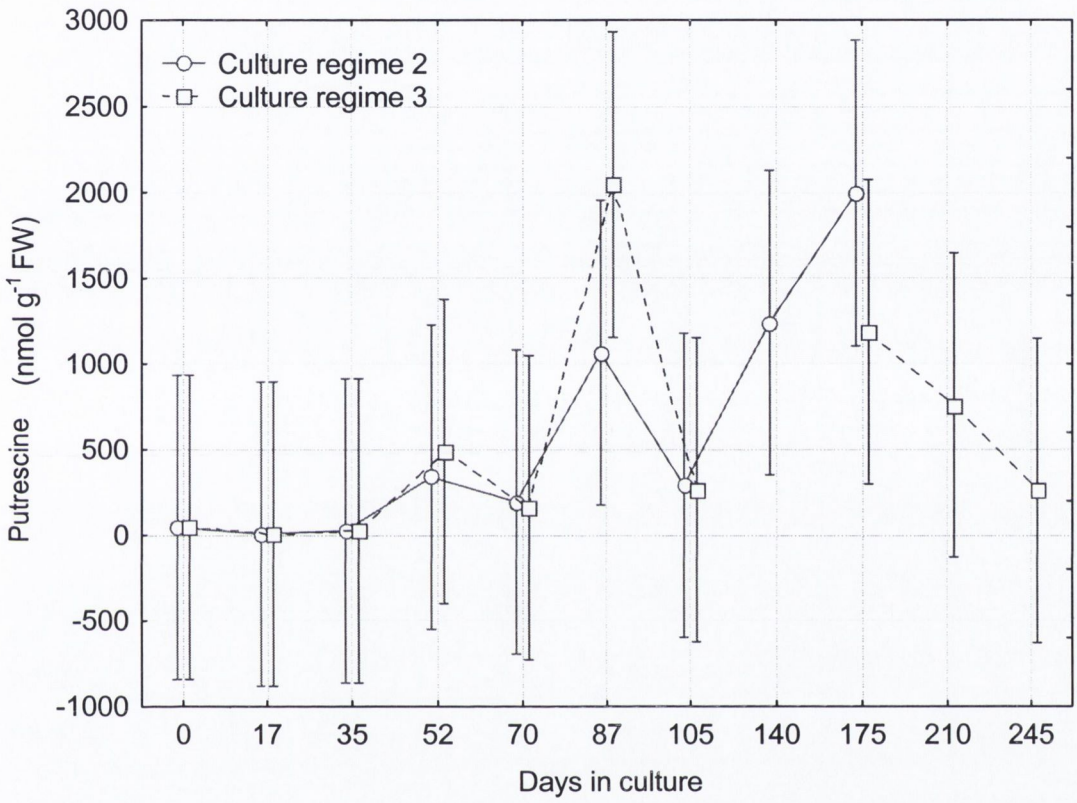


Figure 4. 15. The concentration of putrescine (nmol g^{-1} FW) in leaf samples from shoots of genotype 49 cultured in two culturing regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

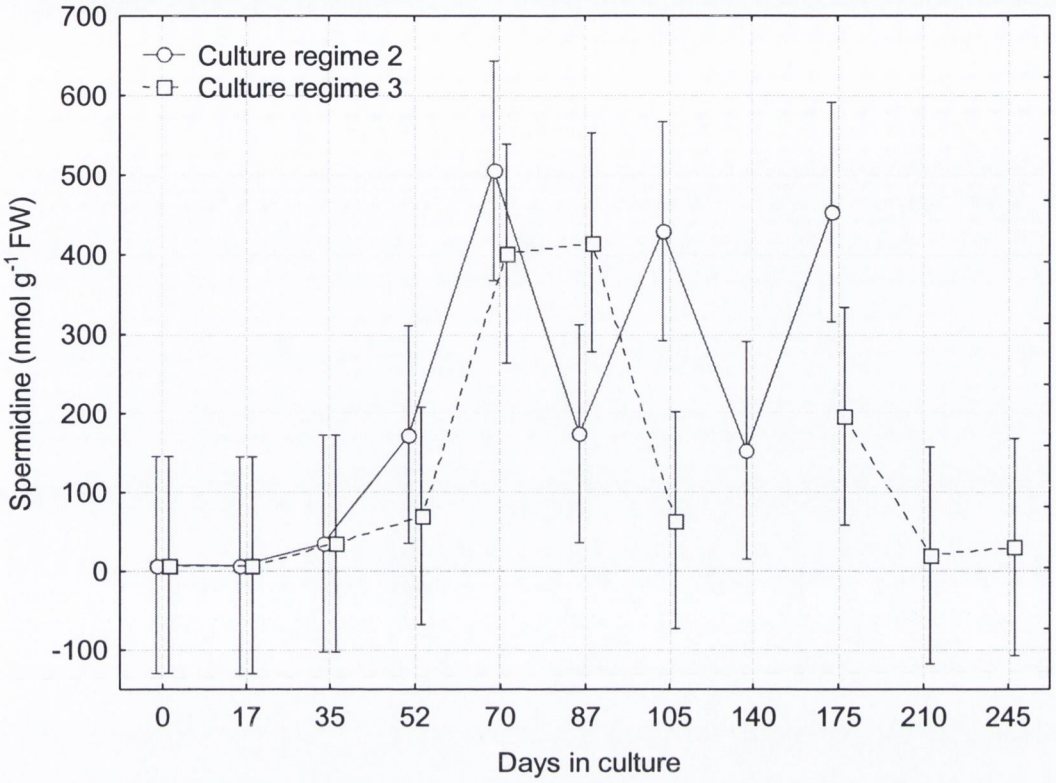


Figure 4. 16. The concentration of spermidine (nmol g^{-1} FW) in leaf samples from shoots of genotype 49 cultured in two culturing regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

The concentration of spermine in culture regime 2 increased significantly from day 0 to day 175 (Tukey's HSD, $p<0.0002$, figure 4. 17). After an initial decrease in concentration following the first transfer to M91 medium, the spermine levels tended to increase with each transfer. In culture regime 3, only the concentration of spermine measured at day 87 (151.4 nmol g^{-1} FW) was significantly different compared to day 0 (Tukey's HSD, $p<0.01$). The concentration of spermine in the leaves tended to increase up to day 87, then decrease until the end of the experiment.

The results in table 4. 4 show that the mean concentration of spermine was significantly higher in culture regime 2 compared to culture regime 3 (One-way ANOVA, $p<0.01$).

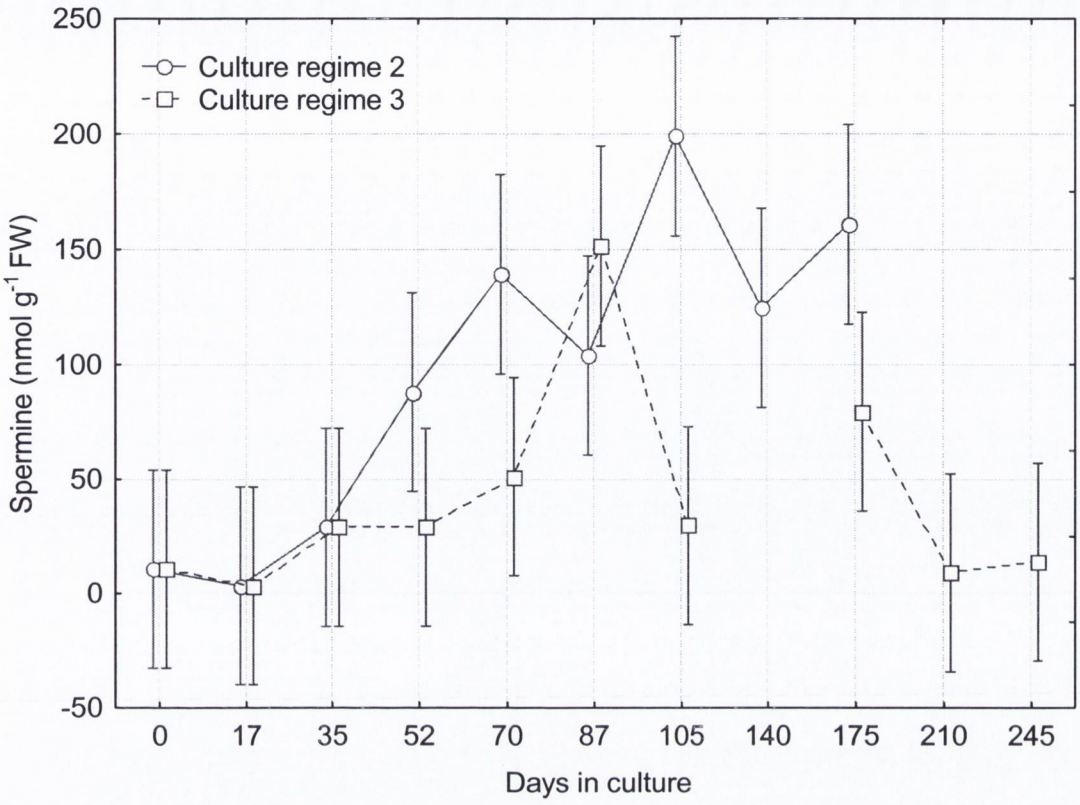


Figure 4. 17. The concentration of spermine (nmol g^{-1} FW) in leaf samples from shoots of genotype 49 cultured in two culturing regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence intervals of the mean of three samples).

(2. 5) Combined analysis

The interaction between ash genotype and the concentration of each of the three polyamines was analysed. In culture regime 1, there was no significant interaction between genotype S11 and F5 for putrescine or spermidine concentration (One-way ANOVA, p=0.06 and p=0.87, respectively). However, the concentration of spermine was significantly different between genotypes in this regime (genotype S11: 8.2±1.7 nmol g^{-1} FW; genotype F5: 3.9±0.5 nmol g^{-1} FW) (One-way ANOVA, p<0.003, table 4. 4).

When the data for all four genotypes was combined and analysed there was no significant difference in the mean putrescine concentration between culture regime 2 and 3 (One-way ANOVA, p=0.70). The concentration of putrescine measured in the four genotypes within and between both culture regimes was similar and no significant interaction occurred (Factorial ANOVA, p=0.98, table 4. 4, figure 4. 18).

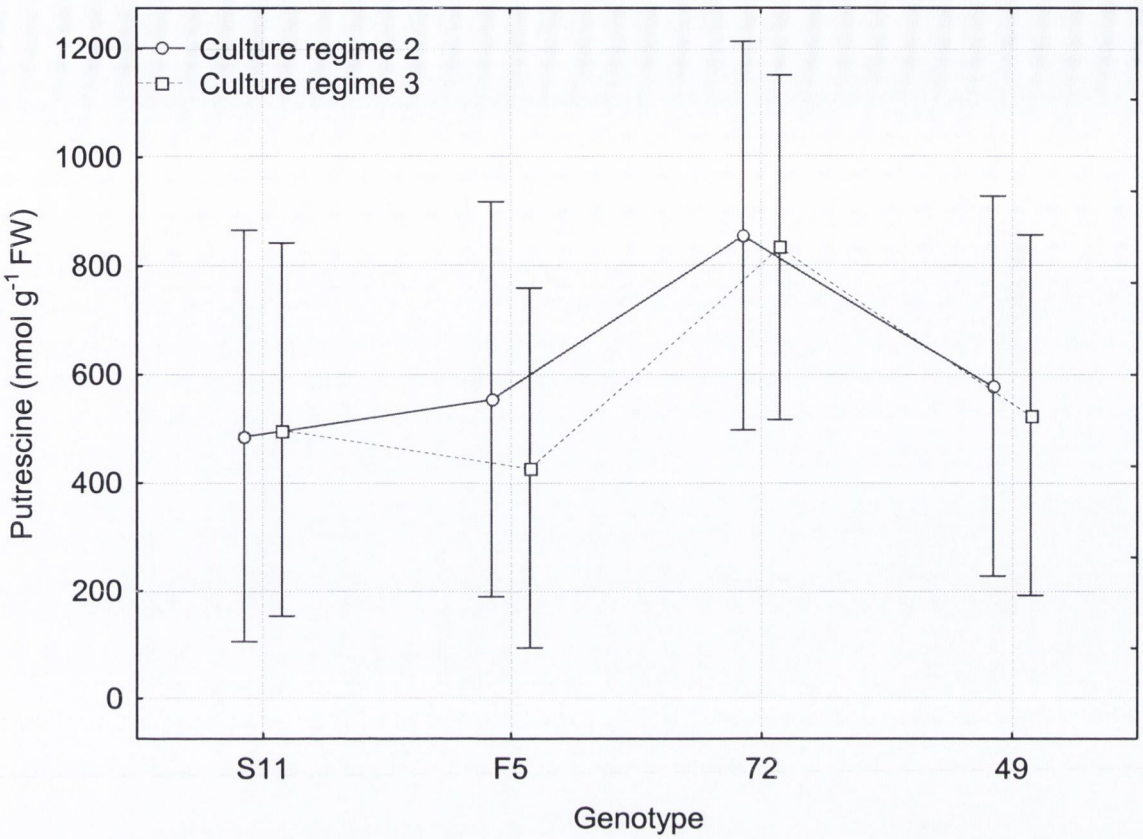


Figure 4. 18. The mean putrescine concentration (nmol g^{-1} FW) for genotype S11, F5, 72 and 49 in two culture regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence interval).

The mean spermidine concentration was significantly higher in culture regime 2 compared to culture regime 3 (One-way ANOVA, $p<0.0000$; figure 4. 19). In culture regime 2 there was a significant difference in spermidine concentration between genotypes (One-way ANOVA, $p<0.0001$), which did not occur in culture regime 3 (One-way ANOVA, $p=0.18$). As a result a significant interaction between genotypes in culture regime 2 and 3 for spermidine concentration was identified (Factorial ANOVA, $p<0.005$, table 4. 4, figure 4. 19).

Spermine concentration was significantly higher in culture regime 2 compared to culture regime 3 (One-way ANOVA, $p<0.00001$). In culture regime 2 there was a significant difference in spermine concentration between genotypes (One-way ANOVA, $p<0.0004$), which also occurred in culture regime 3 (One-way ANOVA, $p=0.009$). In both culture regimes the concentration of spermine was highest in genotype F5 and there was no significant interaction between genotypes in both culture regimes (Factorial ANOVA, $p=0.2$, table 4. 4, figure 4. 20).

The data for the genotypes in each culture regime was combined and is illustrated in figures 4. 21 - 4. 23. In culture regime 1 the concentration of all the polyamines measured was less than 20 nmol g^{-1} FW. In culture regime 2, the concentration of all three polyamines increased with time and by the end of the experiment the concentration of putrescine was over 2000 nmol g^{-1} FW, while the concentration of spermidine and spermine was 361 and 133 nmol g^{-1} FW, respectively. In culture regime 3, the concentration of spermine and spermidine fluctuates slightly but remains below 200 nmol g^{-1} FW for spermidine and below 100 nmol g^{-1} FW for spermine. The concentration of putrescine increases with time and at the end of the experiment is at 1000 nmol g^{-1} FW.

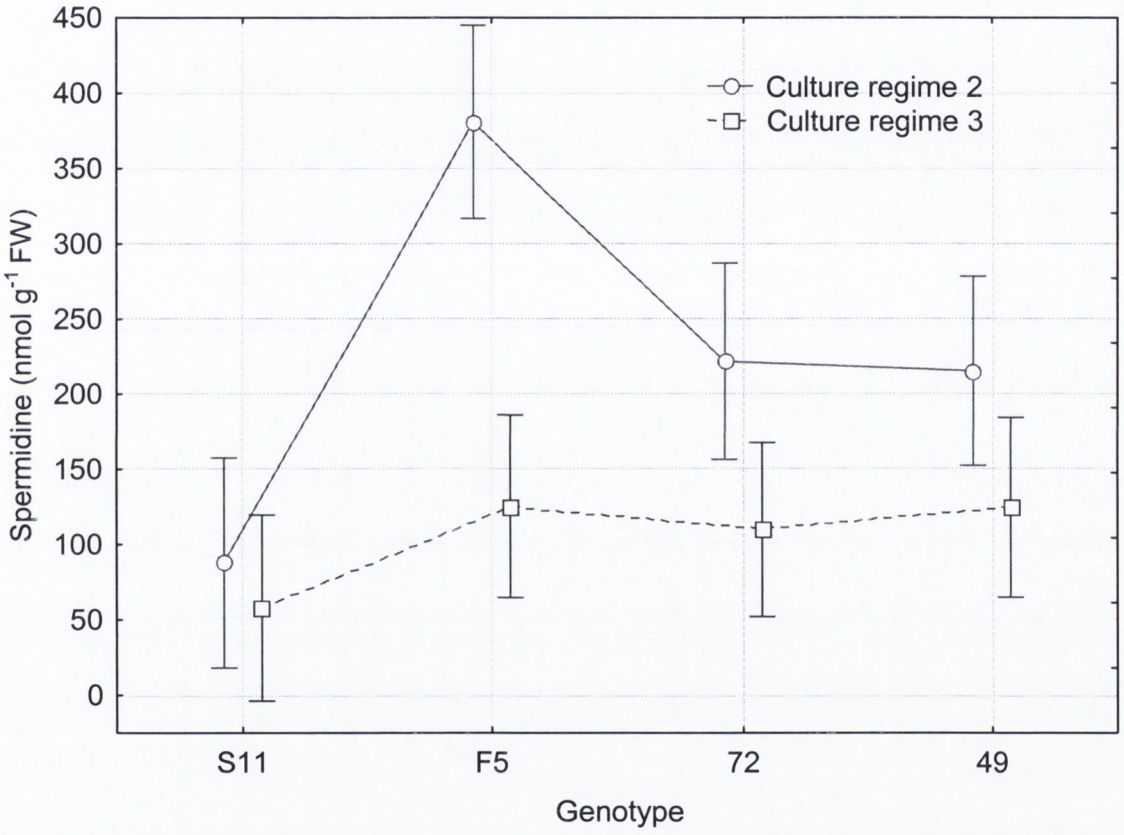


Figure 4. 19. The mean spermidine concentration (nmol g^{-1} FW) for genotype S11, F5, 72 and 49 in two culture regimes (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence interval).

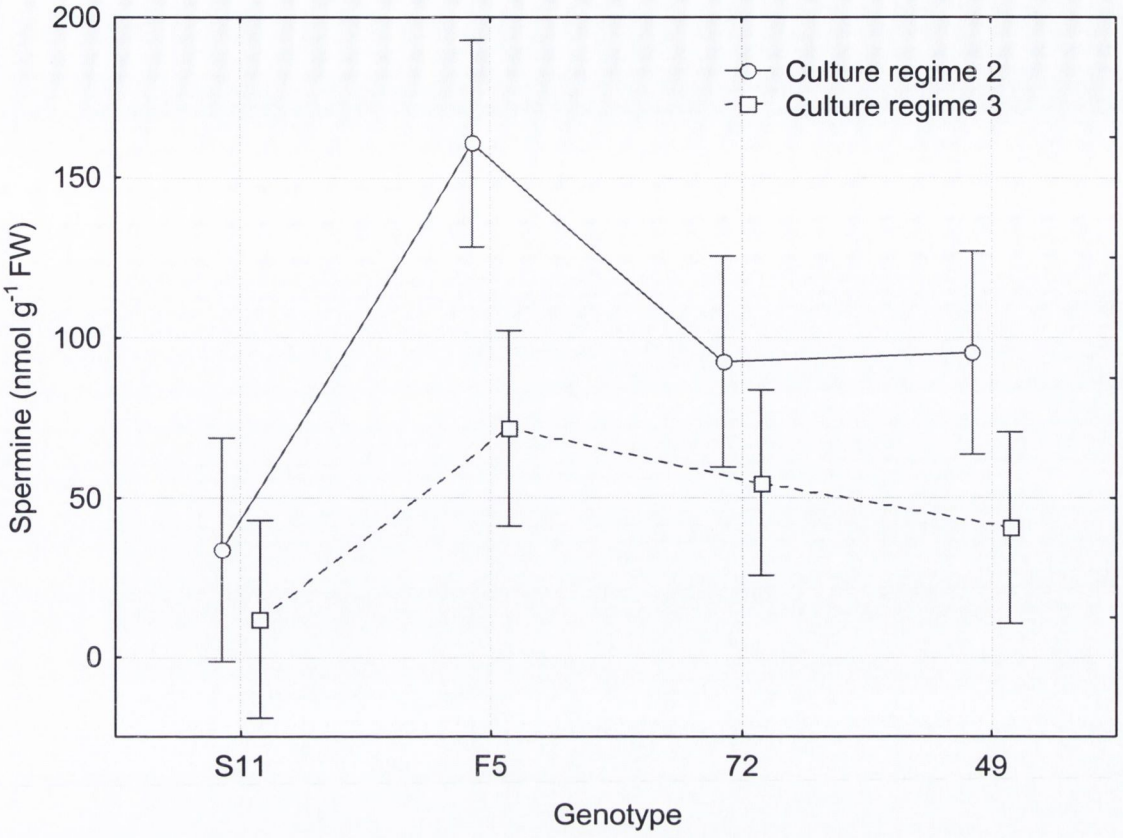


Figure 4. 20. The mean spermine concentration (nmol g^{-1} FW) for genotype S11, F5, 72 and 49 in culture regime 2 and 3 (2: continuous culture on M91; and 3: alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence interval).

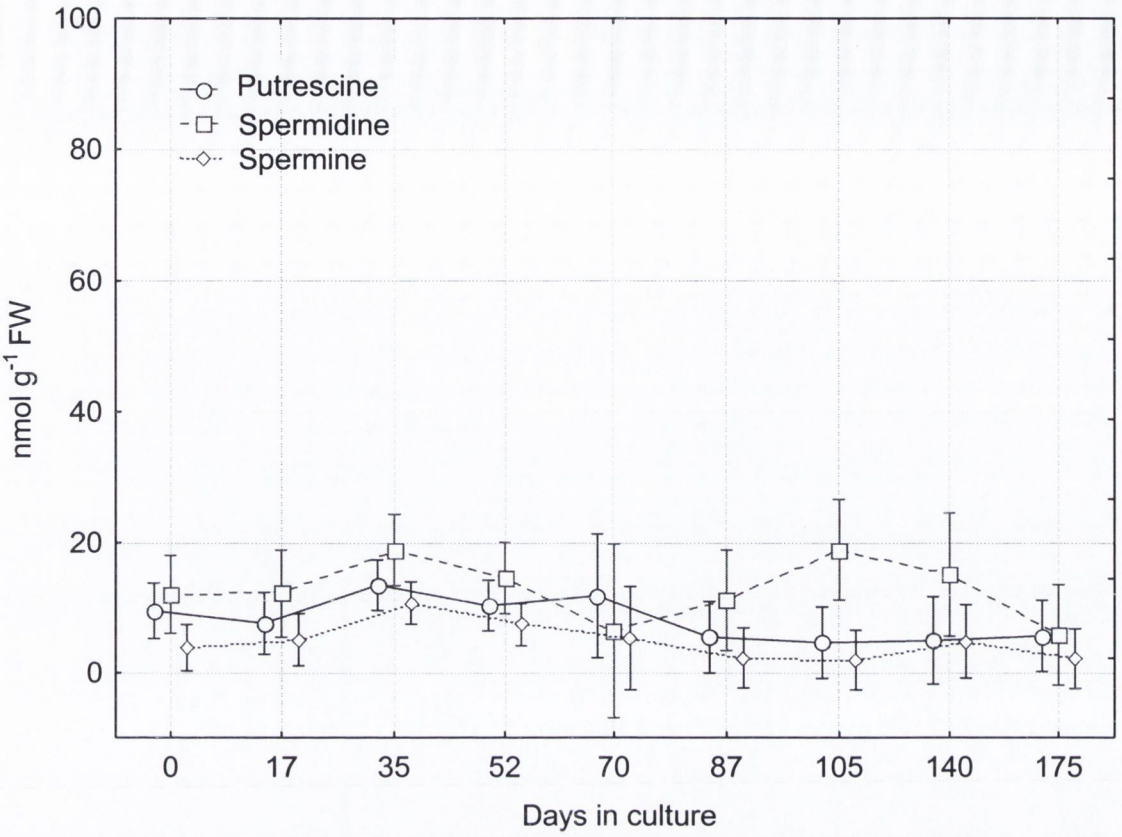


Figure 4. 21. The concentration of polyamines (nmol g^{-1} FW) in culture regime 1 (continuous culture on QRC medium with charcoal) (vertical bars denote the 95% confidence interval).

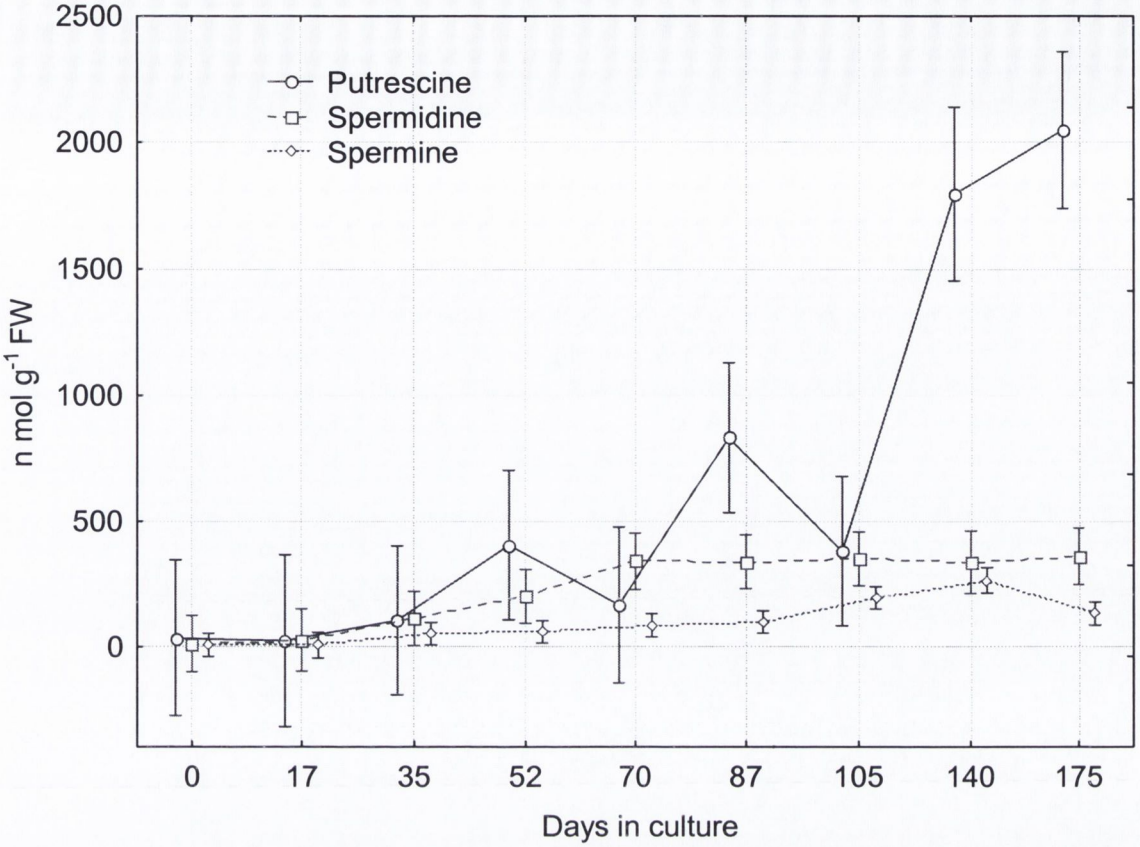


Figure 4. 22. The concentration of polyamines (nmol g^{-1} FW) in culture regime 2 (continuous culture on M91) (vertical bars denote the 95% confidence interval).

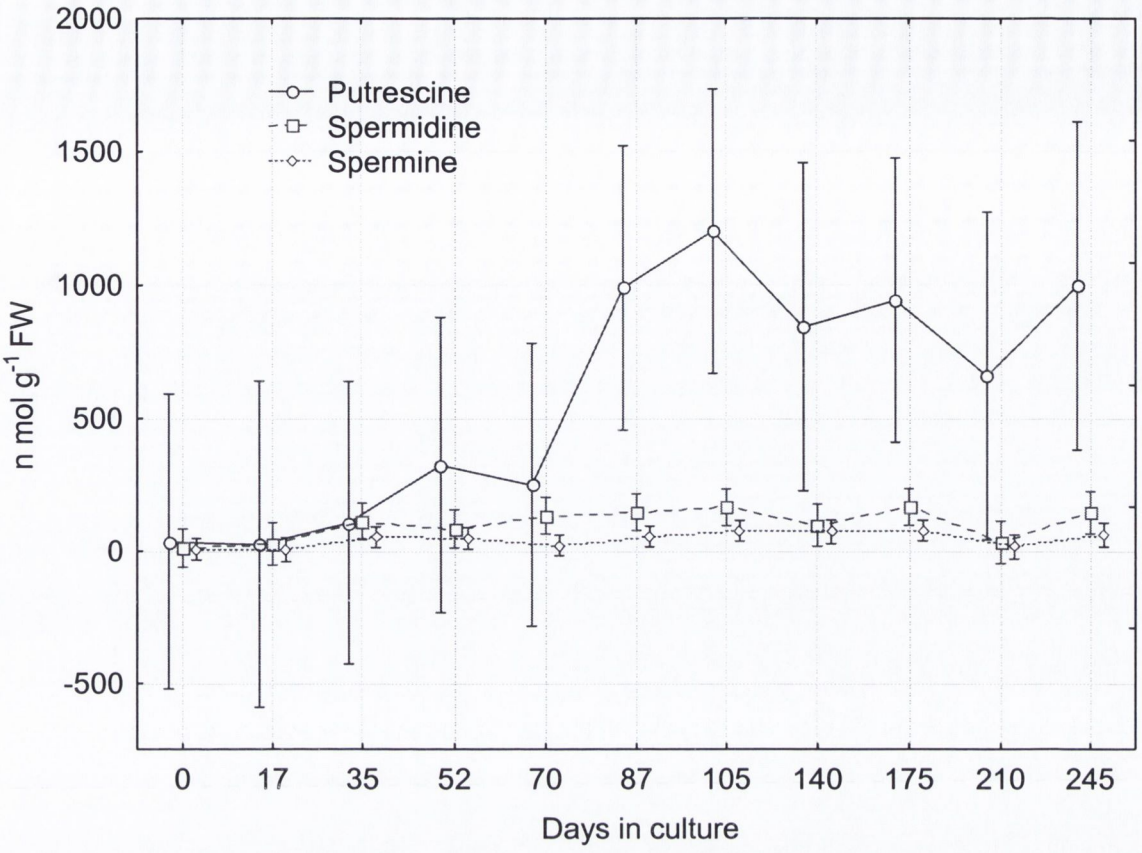


Figure 4. 23. The concentration of polyamines (nmol g^{-1} FW) in culture regime 3 (alternating with M91 and QRC with charcoal) (vertical bars denote the 95% confidence interval).

(2. 1) The effects of four culture media (MS, WPM, DKW and OM) on shoot production.

Single node explants of genotype S8 and selected genotype 47 were used to test the effects of four basic nutrient media; modified MS, modified WPM, DKW and modified OM on shoot proliferation in July 2003 (section 4. 6. E. (2. 1)). In each medium tested the same levels of growth regulators (BAP, TDZ and IBA), sucrose and agar were added. Genotype S8 and 47 had 100% viability in all treatments, except on MS medium where viability in genotype 47 was significantly reduced to 58.3% (Chi square test $p<0.00004$). The number of new shoots produced per node was highest on modified OM medium and lowest on modified MS medium, for both genotypes tested (table 4. 8). Genotype 47 produced more new shoots per node compared to genotype S8, for all the media tested except MS medium. There was no significant interaction between genotype and media for the number of new shoots/ node (Factorial ANOVA, $p=0.26$).

Table 4. 8. Number of new shoots per nodal explant of genotypes S8 and 47 cultured on four types of media (MS, WPM, DKW and OM).

	Genotype		
Media	*S8 (No. new shoots/ node**)*	*47 (No. new shoots/ node**))*	*Total* (No. new shoots/ node**)*
MS	$1.0\pm0.2^{aA***}$	1.0 ± 0.2^{aA}	1.0 ± 0.1^{a}
WPM	1.6 ± 0.2^{aA}	2.2 ± 0.3^{bA}	1.8 ± 0.1^{b}
DKW	1.5 ± 0.2^{aA}	1.8 ± 0.2^{abA}	1.7 ± 0.1^{b}
OM	1.7 ± 0.3^{aA}	2.5 ± 0.2^{bA}	2.0 ± 0.2^{b}

* Data from both genotypes combined. ** ± Standard error. *** Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.03$) according to Tukey's unequal N HSD.

The mean shoot length (mm) of the new shoots was longest for those produced on modified OM and shortest for those produced on modified WPM medium (table 4. 9). Genotype S8 produced significantly longer shoots compared to genotype 47 (5.9 and 2.7 mm, respectively) (One-way ANOVA, $p<0.0002$), the interaction between media and genotype for mean shoot length was significant (Factorial ANOVA, $p<0.00004$) (table 4. 9, figure 4. 24).

Table 4. 9. Mean shoot length (mm) of new shoots produced by nodal explants of genotype S8 and 47 cultured on MS, WPM, DKW or OM.

	Genotype		
Media	*S8 (Mean shoot length (mm))***	*47 (Mean shoot length (mm))***	*Total* (Mean shoot length (mm))***
MS	$3.2\pm0.5^{aA***}$	2.5 ± 0.6^{aA}	2.9 ± 0.4^{a}
WPM	2.10 ± 0.5^{aA}	1.8 ± 0.2^{aA}	1.9 ± 0.3^{a}
DKW	5.47 ± 1.7^{abA}	2.8 ± 0.3^{aA}	3.6 ± 0.6^{a}
OM	11.0 ± 1.4^{bA}	3.1 ± 0.2^{aB}	7.5 ± 1.0^{b}

* Data from both genotypes combined. ** ± Standard error. *** Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.01$) according to Tukey's unequal N HSD.

Figure 4. 24. Shoots of genotype S8 (left hand side) and genotype 47 (right hand side) cultured on modified OM medium.

For both genotypes, the propagation rate was highest from explants cultured on modified OM compared to all the other media tested (table 4. 10). There was no significant difference in propagation rate, over all the media, between genotype S8 and 47 (2.2 and 2.4 new explants/ explant, respectively). The interaction between media and genotype for propagation rate was significant (Factorial ANOVA, $p<0.02$).

Table 4. 10. The propagation rate of nodal explants of genotype S8 and 47 cultured on four types of media (MS, WPM, DKW and Ruigini OM).

	Genotype		
	S8	*47*	*Total**
Media	*(Propagation rate**)*	*(Propagation rate**)*	*(Propagation rate**)*
MS	1.45±0.3^{aA***}	2.35±0.3^{aA}	1.8±0.2^{a}
WPM	1.77±0.2^{aA}	2.31±0.2^{aA}	2.0±0.1^{a}
DKW	2.05±0.2^{aA}	2.17±0.1^{aA}	2.1±0.1^{a}
OM	3.28±0.2^{bA}	2.78±0.2^{aA}	3.1±0.1^{b}

* Data from both genotypes combined. ** ± Standard error. *** Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.03$) according to Tukey's HSD.

In genotype S8, there was a significant difference for the increase in dry weight of explants on modified OM medium compared to modified MS medium (Tukey's unequal N HSD, $p<0.02$) (table 4. 11). In genotype 47, there was no significant difference for the increase in dry weight between the four media tested (One-way ANOVA, $p=0.56$) (table 4. 11). A significant difference in the overall dry weight increase was identified between genotypes S8 and 47 (723.4 and 1575.6%, respectively), (ANOVA, $p<0.02$). There was no interaction between media and genotype for percentage dry weight increase (Factorial ANOVA, $p<0.73$).

Table 4. 11. Dry weight increase (%) of genotypes S8 and selected genotype 47 cultured on four types of media (MS, WPM, DKW and OM).

	Genotype		
	S8	*47*	*Total**
Media	*(DW increase (%)**)*	*(DW increase (%)**)*	*(DW increase (%)**)*
MS	397.9±136.1^{a***}	1413.1±618.4^{a}	818±278^{a}
WPM	510.3±147.1^{ab}	757.1±164.4^{a}	605.8±110.8^{a}
DKW	852.2±283.8^{ab}	1486.6±497.7^{a}	1275.2±345.9^{a}
OM	1106.4±220.3^{b}	2318.5±864.7^{a}	1651.8±412.7^{a}

* Data from both genotypes combined. ** ± Standard error. *** Different letters within columns indicate a significant difference ($p<0.05$) according to Tukey's unequal N HSD.

The experiment was repeated in November 2003 using selected genotype 49 to test all four media; genotype S8 to test modified WPM and DKW; and genotype F5 to test modified MS and modified OM.

For genotype 49, the viability rate for all media tested was over 95%. There was no significant difference in the number of new shoots produced per node growing on any of the four media tested (One-way ANOVA, $p=0.07$) (table 4. 12). However, there was a significant difference in the mean shoot length of the new shoots produced (One-way ANOVA, $p<0.000003$); nodes growing on the modified OM medium produced the longest shoots (table 4. 12). The propagation rate was significantly higher on modified OM medium compared to modified MS medium and DKW medium (Tukey's unequal N HSD, $p<0.05$) (table 4. 12). There was a significant difference in the increase in dry weight of the explants on all four media, the largest increase recorded on modified MS medium and modified OM medium (One-way ANOVA, $p<0.01$) (table 4. 12).

Table 4. 12. Number of new shoots per node, mean shoot length (mm), propagation rate and dry weight increase (%) for explants of genotype 49 cultured on four types of media (MS, WPM, DKW and OM).

Media	*New shoots/ node**	*Mean shoot length (mm)**	*Propagation rate**	*Dry weight increase (%)**
MS	1.6±0.2[a]	3.7±0.5[ab]	1.9±0.2[a]	1437.5±173.0[ab]
WPM	2.4±0.2[a]	2.6±0.2[bc]	2.3±0.2[ab]	933.3±63.6[ab]
DKW	1.8±0.2[a]	1.6±0.1[c]	1.9±0.2[a]	1000.0±136.1[a]
OM	2.3±0.3[a]	4.6±0.5[a]	2.8±0.2[b]	1412.5±90.9[b]

* ± Standard error. ** Different letters within columns indicate a significant difference (mean shoot length, $p<0.004$; propagation rate, $p<0.05$; dry weight increase, $p<0.03$) according to Tukey's unequal N HSD.

The results for genotype S8, cultured on modified WPM and DKW, and F5 cultured on modified MS and OM media are not presented in a table but are described in the following two paragraphs.

The viability rate was over 90% for nodes of genotype S8 cultured on modified WPM and DKW. The number of new shoots per node was significantly higher on modified WPM medium compared to DKW medium (2.2 and 1.6 new shoots/ node, respectively) (One-way ANOVA, $p<0.03$). However, mean shoot length was higher on DKW medium than on modified WPM (2.8mm and 1.9mm, respectively) (One-way ANOVA, $p<0.03$). There was no significant difference in propagation rate from the two media tested (modified WPM: 2.25, DKW: 1.95) (One-way ANOVA, $p=0.18$). Explants on modified WPM had a significantly higher percentage increase in dry weight compared to those growing on DKW medium (1241.7% and 1004.2%, respectively) (One-way ANOVA, $p<0.03$).

Nodes of genotype F5 cultured on modified MS and modified OM medium had a 100% viability rate. The number of new shoots per node was similar on modified MS medium compared to modified OM medium (1.8 and 2.2 new shoots/ node, respectively) (One-way ANOVA, p=0.12). The mean shoot length of the new shoots was similar for both media tested (modified MS: 4.8mm, modified OM: 4.7mm) (One-way ANOVA, p=0.96), as was the propagation rate (modified MS: 2.2, modified OM: 2.5) (One-way ANOVA, p=0.26), and the increase in dry weight of the nodal explants (modified MS: 2920.9%, modified OM: 2641.7%) (One-way ANOVA, p=0.42).

(2. 2) The effects of carbon source on shoot production.

In September 2003, genotype S8 and selected genotype 49 were used to test media with four different sources of carbohydrate; sucrose, glucose, mannitol and an equimolar mixture of sucrose and mannitol (section 4. 6. E. (2. 2)). Contamination levels in this experiment were high and affected explants were removed from the experiment, consequently some treatments have low numbers of replicates and were not statistically analysed. It was noted that fungal contamination was particularly high in the media containing glucose.

The viability rate for genotype S8 was significantly different over the four media tested (Chi square test, $p<0.04$), and was highest on the media containing mannitol on its own or mannitol combined with sucrose (table 4. 13). There was no significant difference in the number of new shoots produced by nodes growing on the different carbohydrate media tested (One-way ANOVA, p=0.2). Of the new shoots produced, those growing on the sucrose media were the longest (statistical tests not done on this variable due to the low numbers of replicates). There was a significant difference in propagation rate, explants from the sucrose media produced the most new explants (One-way ANOVA, $p<0.04$) (glucose treatment excluded from the analysis due to low replicate numbers). There was no significant difference in the percentage dry weight increase between the four carbohydrate media (One-way ANOVA, p=0.1) (glucose treatment excluded from the analysis due to low replicate numbers).

Table 4. 13. Viability (%), number of new shoots per node, mean shoot length (mm), propagation rate and dry weight increase (%) for explants of genotype S8 cultured on M91 media containing four different carbohydrate sources.

Carbohydrate	*Viability (%)*	*New shoots/ node**	*Mean shoot length (mm)**	*Propagation rate**	*Dry weight increase (%)**
Sucrose	52.6	1.2±0.2 [a]**	3.0	1.4±0.3[a]	313.5±65.4[a]
Glucose	50	0.6±0.2 [a]	1.5	0.2±0.1	162.9±52.9
Mannitol	79.2	0.8±0.2 [a]	2.0	0.7±0.2[a]	196.2±50.7[a]
Suc/ Man***	82.6	0.9±0.2 [a]	1.9	0.6±0.2[b]	169.4±32.1[a]

* ± Standard error. ** Different letters within columns indicate a significant difference ($p<0.05$) according to Tukey's unequal N HSD. *** Equimolar mixture of sucrose and mannitol.

For genotype 49, the sucrose/ mannitol (suc/ man) mixture treatment was excluded from analysis as only two individuals remained alive after losses due to contamination and explant death. There was no significant difference in viability between the three remaining media tested (Chi square test, $p=0.5$) (table 4. 14). There was no significant difference in the number of new shoots produced by nodes in the different treatments (One-way ANOVA, $p=0.3$). Mean shoot length was not analysed due to the low replicate numbers of the treatments. There was no significant difference in propagation rate or percentage dry weight increase between the three media tested (One-way ANOVA, $p=0.2$, for both variables tested).

Table 4. 14. Viability (%), number of new shoots per node, mean shoot length (mm), propagation rate and dry weight increase (%) for explants of genotype 49 cultured on media containing four different carbohydrate sources.

Carbohydrate	*Viability (%)*	*New shoots/ node**	*Mean shoot length (mm)**	*Propagation rate**	*Dry weight increase (%)**
Sucrose	57.9	1.1±0.2 [a]**	1.7	1.1±0.3[a]	389.8±90.7[a]
Glucose	37.5	0.7±0.2 [a]	1.2	0.7±0.2[a]	210.4±33.5[a]
Mannitol	40	0.8±0.2 [a]	0.7	0.4±0.3[a]	173.1±54.1[a]
Suc/ Man***	-	-	-	-	-

* ± Standard error. ** Different letters within columns indicate a significant difference ($p<0.05$) according to Tukey's unequal N HSD. *** Equimolar mixture of sucrose and mannitol

The experiment was repeated in January 2004 using genotypes S11 and selected genotype 49 to test the four carbohydrate media. Contamination was not a problem in this experiment. For genotype S11, viability was significantly different between the four sugar types tested and was lowest on the sucrose medium (One-way ANOVA, $p<0.02$) (table 4. 15). There was no significant difference in the number of new shoots produced by nodes, the mean shoot length of the new shoots or the propagation rate between the four treatments (One-way ANOVA, 0.32, $p=0.2$ and $p=0.2$ respectively). The percentage dry weight increase of explants in different treatments was significantly different, the largest increase in dry matter was on the sucrose/ mannitol media (One-way ANOVA, $p<0.01$).

Table 4. 15. Viability (%), number of new shoots per node, mean shoot length (mm), propagation rate and dry weight increase (%) for explants of genotype S11 cultured on media containing four different carbohydrate sources.

Carbohydrate	*Viability (%)*	*New shoots/ node**	*Mean shoot length (mm)**	*Propagation rate **	*Dry weight increase (%)**
Sucrose	83.3^{a**}	1.4 ± 0.2^{a}	1.9 ± 0.2^{a}	1.3 ± 0.2^{a}	409.7 ± 81.6^{ab}
Glucose	100^{b}	1.5 ± 0.1^{a}	1.6 ± 0.1^{a}	1.5 ± 0.1^{a}	410.5 ± 65.4^{ab}
Mannitol	96.7^{ab}	1.6 ± 0.1^{a}	1.7 ± 0.1^{a}	1.6 ± 0.1^{a}	227.8 ± 35.2^{a}
Suc/ Man***	100^{b}	1.8 ± 0.2^{a}	2.2 ± 0.2^{a}	1.8 ± 0.2^{a}	767.6 ± 139.7^{b}

* ± Standard error. ** Different letters within columns indicate a significant difference (viability, $p<0.03$; dry weight increase, $p<0.01$) according to Tukey's unequal N HSD. *** Equimolar mixture of sucrose and mannitol.

Viability was low over all treatments for genotype 49, the highest viability rate was recorded on the media containing mannitol as the sole carbohydrate source (One-way ANOVA, $p<0.003$) (table 2. 9). Due to low viability rates it was not possible to statistically test the remaining variables reported in table 4. 16.

Table 4. 16. Viability (%), number of new shoots per node, mean shoot length (mm), propagation rate and dry weight increase (%) for explants of genotype 49 cultured on media containing four different carbohydrate sources.

Carbohydrate	*Viability (%)*	*New shoots/ node**	*Mean shoot length (mm)**	*Propagation rate**	*Dry weight increase (%)**
Sucrose	23.8^{ab}**	1.2	2.9	1.2	422.2
Glucose	3.6^{b}	0.8	2.2	0.6	223.5
Mannitol	32.1^{a}	1.0	2.0	1.0	25.0
Suc/ Man***	3.6^{b}	2.0	2.50	2.0	0.0

* ± Standard error. ** Different letters within columns (lowercase) indicate a significant difference (viability, $p<0.01$) according to Tukey's unequal N HSD. ***Equimolar mixture of sucrose and mannitol

(2. 3) The effects of different combinations of the cytokinins BAP and TDZ on shoot production.

Single node explants of selected genotypes 8 and 49 were used to test the effect of different combinations of BAP (0, 1 or 5mg/l) and TDZ (0, 0.25, 0.5mg/l) on shoot production (section 4. 6. E. (2. 3)). For both genotypes viability was significantly different between all nine media tested (One-way ANOVA, genotype 8: $p<0.02$ and genotype 49: $p<0.0003$, respectively) (table 4. 17). Overall, viability was significantly lower in genotype 49 compared to genotype 8 (69% and 84% viability respectively) (Chi square test, $p<0.001$).

Table 4. 17. The viability (%) of single node explants of selected genotypes 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.

Media code	*[BAP] (mg/l)*	*[TDZ] (mg/l)*	*Genotype 8 Viability (%)*	*Genotype 49 Viability (%)*
A	5	0.5	85^{ab*}	56.2^{abc}
B	5	0.25	70^{ab}	91.7^{a}
C	5	0	100^{ab}	70.8^{abc}
D	1	0.5	90^{ab}	83.3^{a}
E	1	0.25	83.3^{ab}	28.6^{b}
F	1	0	61.1^{b}	88.9^{a}
G	0	0.5	94.4^{ab}	81.2^{ac}
H	0	0.25	100^{a}	75.0^{abc}
I	0	0	75^{b}	41.7^{bc}

* Different letters within columns (lowercase) indicate a significant difference ($p<0.03$) according to Tukey's unequal N HSD.

The data for the number of new shoots per node (for both genotypes) were normalised by square root transformation prior to analysis. For both genotypes the number of new shoots per node was significantly different between all nine media tested (One-way ANOVA, genotype 8: $p<0.001$, genotype 49: $p<0.001$) (table 4. 18). The most new shoots were produced on media with 5mg/l BAP and 0.5mg/l TDZ for genotype 8 and media with 5mg/l BAP and 0.25mg/l TDZ for genotype 49. Explants cultured on the high cytokinin media tended to form clumps of multiple short shoots and callus (figure 4. 25).

Table 4. 18. The number of new shoots produced per node of selected genotype 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.

Media code	*[BAP] (mg/l)*	*[TDZ] (mg/l)*	*Genotype 8 New shoots/ node ± S. E.*	*Genotype 49 New shoots/ node ± S. E.*
A	5	0.5	$2.9\pm0.6^{a*}$	2.4 ± 0.7^{abc}
B	5	0.25	2.2 ± 0.9^{abc}	4.6 ± 0.7^{b}
C	5	0	0.9 ± 0.2^{abc}	1.6 ± 0.3^{ac}
D	1	0.5	1.5 ± 0.3^{ab}	1.9 ± 0.3^{abc}
E	1	0.25	1.5 ± 0.4^{abc}	0.6 ± 0.2^{ad}
F	1	0	0.4 ± 0.2^{bc}	0.6 ± 0.2^{ad}
G	0	0.5	1.5 ± 0.2^{a}	2.9 ± 0.5^{bc}
H	0	0.25	1.8 ± 0.2^{a}	2.9 ± 0.9^{abc}
I	0	0	0.2 ± 0.1^{c}	0.1 ± 0.1^{d}

* Different letters within columns (lowercase) indicate a significant difference ($p<0.02$) according to Tukey's unequal N HSD.

Figure 4. 25. Two single node explants of genotype 8 cultured on medium containing 5mg/l BAP and 0.5mg/l TDZ.

The length of shoots produced per node as well as the propagation rate was recorded (table 4. 19 and 4. 20). For genotype 8, the mean shoot length of the new shoots was similar between the nine media tested, however media with 5mg/l BAP and 0.5mg/l TDZ produced significantly longer shoots than media with 0mg/l BAP and 0.5mg/l TDZ (Tukey's unequal N HSD, $p<0.04$) (table 4. 19). There was no significant difference in mean shoot length between the different media for genotype 49, the longest shoots being produced on media with 1mg/l BAP and 0.5mg/l TDZ (One-

way ANOVA, $p<0.07$). There was a significant difference between the propagation rate of single node explants from different media in both genotypes (One-way ANOVA, genotype 8: $p<0.00$; genotype 49: $p<0.00$) (table 4. 20). The highest propagation rate for genotype 8 was highest on media with 5mg/l BAP and 0.5mg/l TDZ and the highest propagation rate for genotype 49 was on media with 5mg/l BAP and 0.25mg/l TDZ (table 4. 20). Percentage dry weight increase was significantly different, for both genotypes, between the nine media tested (One-way ANOVA, genotype 8: $p<0.03$; genotype 49: $p<0.01$). For genotype 8, the increase in dry weight was largest on media with 5mg/l BAP and 0.5mg/l TDZ and for genotype 49 the increase was largest on media with 0mg/l BAP and 0.25mg/l TDZ, although measurements varied a lot on this media (table 4. 21).

Table 4. 19. The mean shoot length (mm) of the new shoots produced by nodes of selected genotype 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.

Media code	*[BAP] (mg/l)*	*[TDZ] (mg/l)*	*Genotype 8 Mean shoot length (mm) ± S. E.*	*Genotype 49 Mean shoot length (mm) ± S. E.*
A	5	0.5	$2.6\pm0.3^{a*}$	2.6 ± 0.5^{a}
B	5	0.25	1.9 ± 0.3^{ab}	2.9 ± 0.2^{a}
C	5	0	1.2 ± 0.2^{ab}	2.1 ± 0.3^{a}
D	1	0.5	2.2 ± 0.3^{ab}	3.4 ± 0.5^{a}
E	1	0.25	1.7 ± 0.4^{ab}	1.9 ± 0.1^{a}
F	1	0	1.0 ± 0.0^{ab}	1.3 ± 0.3^{a}
G	0	0.5	1.4 ± 0.1^{b}	2.3 ± 0.2^{a}
H	0	0.25	1.8 ± 0.4^{ab}	2.9 ± 0.5^{a}
I	0	0	1.0 ± 0.0^{ab}	2.0 ± 1.0^{a}

* Different letters within columns (lowercase) indicate a significant difference ($p<0.04$) according to Tukey's unequal N HSD.

Table 4. 20. The propagation rate of nodal explants of selected genotype 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.

Media code	*[BAP] (mg/l)*	*[TDZ] (mg/l)*	*Genotype 8 Propagation rate ± S. E.*	*Genotype 49 Propagation rate ± S. E.*
A	5	0.5	$2.9\pm0.6^{a*}$	3.8 ± 0.9^{ab}
B	5	0.25	2.7 ± 1.1^{abd}	4.6 ± 0.7^{a}
C	5	0	0.8 ± 0.2^{bc}	1.6 ± 0.3^{bc}
D	1	0.5	1.6 ± 0.4^{abcd}	2.6 ± 0.4^{abc}
E	1	0.25	1.6 ± 0.4^{abcd}	3.0 ± 1.0^{abc}
F	1	0	0.4 ± 0.2^{c}	2.0 ± 0.6^{abc}
G	0	0.5	1.4 ± 0.2^{cd}	3.2 ± 0.5^{abc}
H	0	0.25	1.5 ± 0.2^{abcd}	3.8 ± 0.9^{ab}
I	0	0	0.2 ± 0.1^{c}	0.3 ± 0.2^{c}

* Different letters within columns (lowercase) indicate a significant difference ($p<0.03$) according to Tukey's unequal N HSD.

Table 4. 21. The dry weight increase (%) of nodal explants of selected genotype 8 and 49 cultured on nine different media containing different combinations of the cytokinins BAP and TDZ.

Media code	*[BAP] (mg/l)*	*[TDZ] (mg/l)*	*Genotype 8 Propagation rate ± S. E.*	*Genotype 49 Propagation rate ± S. E.*
A	5	0.5	1814.2±352.3[a]*	1472.2±245.7[a]
B	5	0.25	1057.1±346.4[ab]	1289.4±176.5[a]
C	5	0	456.5±81.0[ab]	705.9±114.6[a]
D	1	0.5	1005.1±266.8[ab]	980.6±106.4[a]
E	1	0.25	520.8±107.4[ab]	529.2±139.5[a]
F	1	0	1081.1±264.9[ab]	655.7±147.4[a]
G	0	0.5	619.1±153.5[ab]	886.5±187.8[a]
H	0	0.25	509.3±95.1[ab]	1793.5±866.6[a]
I	0	0	525.9±100.4[b]	855.8±242.4[a]

* Different letters within columns (lowercase) indicate a significant difference ($p<0.03$) according to Tukey's unequal N HSD.

(3) The effects of culturing on modified MS medium compared to modified OM medium on shoot production.

Genotype S11 was used to test the effect of two culture regimes using modified MS and modified OM medium (section 4. 6. E. (3)). The two regimes involved either culturing continuously on modified MS or modified OM medium, or alternating culture the media with QRC media and activated charcoal at every second transfer. The results are shown in table 4. 22. The mean viability rate was between 80 and 90% for all treatments. Culture regime had no effect on the mean propagation rate for explants cultured on modified MS media or modified OM media (One-way ANOVA, $p=0.65$ and $p=0.06$, respectively). At the third transfer period shoots from the modified OM media continuous culture regime could not be transferred due to substantial bacterial contamination and loss of culture vigour.

Table 4. 22. The propagation rates for genotype S11 in two culture regimes set up to test the effect of culturing on modified MS medium compared to modified OM medium.

Medium	*Modified MS*		*Modified OM*	
Transfer**	*Culture regime**		*Culture regime**	
	Alternate	Continuous	Alternate	Continuous
Transfer 1 ± S. E.***	2.2±0.1^{a}****		2.1±0.1^{Aa}	
Transfer 2 ± S. E.	3.3±0.5^{Aa}	3.0±0.4^{Aa}	1.9±0.2^{Aab}	2.2±0.3^{Aa}
Transfer 3 ± S. E.	1.6±0.1^{Ab}	2.1±0.1^{Aa}	1.6±0.1^{b}	-
Mean ± S. E.	2.4±0.1^{A}	2.4±0.2^{A}	2.1±0.1^{A}	1.9±0.1^{A}

* Alternate culture: alternates with QRC media with charcoal for every second transfer; continuous culture: cultures are transferred to the same medium at each transfer. ** All explants were divided into separate regimes after transfer 1. *** ± Standard error. **** Different letters between rows (lowercase) in the same column or between columns (uppercase) in the same media type indicate a significant difference according to Tukey's HSD at the 5% level.

Using the same experimental set up, genotype 49 was used to test the effect of the same two culture regimes using modified MS and modified OM medium. The results are shown in table 4. 23. The mean viability rate was over 90% for all treatments except the continuous culture regime on modified MS medium in which only 58% of explants were viable. Culture regime had no effect on the mean propagation rate overall transfers for explants cultured on modified MS media (One-way ANOVA, $p=0.11$). Culture regime had a significant effect on the propagation rate for shoots cultured on the modified OM media. Propagation rates were significantly higher for those in the continuous culture regime (One-way ANOVA, $p<0.0004$). However, it was noted that shoots from the continuous culture regime had excess callus growth, and were suffering from physiological disorders including vitrification and shoot fasiation, in many cases it was difficult to discern if shoots were of adventitious origin arising from the callus or if they were produced by axillary buds on the explant (figure 4. 26).

Figure 4. 26. Explants of genotype 49 suffering from excess callus growth and spindly vitrified leaves.

As a further investigation, after the second transfer period shoots from genotype 49 were taken from modified MS and modified OM media in the alternate culture regimes and tested for rooting capacity. Shoots from the continuous treatment were not suitable for rooting and no shoots from genotype S11 were considered suitable for root induction i.e. shoots were very small (<3mm long). There was no significant difference in the percentage of shoots rooting from modified MS (50%) or modified OM (60%) media (Chi-square test, p=0.55).

Table 4. 23. The propagation rates for genotype 49 in two culture regimes to test the effect of culturing on modified MS medium compared to modified OM medium.

	Modified MS		*Modified OM*	
	*Culture regime**		*Culture regime**	
*Transfer***	Alternate	Continuous	Alternate	Continuous
Transfer 1 ± S. E.***	2.5±0.1^{a}****		3.5±0.1^{Aa}	
Transfer 2 ± S. E.	3.0±0.3 ^{Aa}	2.6±0.4 ^{Aa}	3.6±0.2^{Aa}	3.2±0.1^{Aa}
Transfer 3 ± S. E.	3.0±0.1 ^{Aa}	2.7±0.3 ^{Aa}	3.3±0.2^{Aa}	7.4±0.7^{Bb}
Mean ± S. E.	2.9±0.1^{A}	2.5±0.2^{A}	3.5±0.1^{A}	4.7±0.3^{B}

*Alternate culture: alternates with QRC medium with charcoal every second transfer; continuous culture: cultures transferred to the same medium at each transfer. **All explants were divided into two regimes after transfer 1. *** ± Standard error. ****Different letters between rows (lowercase) in the same column or between columns (uppercase) in the same media type indicate a significant difference according to Tukey's HSD at the 5% level.

(4) *The effects of culturing on modified MS and modified OM type A compared to modified MS and modified OM type B on shoot production and health.*

Explants of genotype 54 were used to test the effect of culturing on modified MS type A and B and modified OM type A and type B (section 4. 6. E. (4)). The results are shown in table 4. 24. Shoots cultured on modified MS medium type B had significantly lower viability rates compared to those cultured on type A (One-way ANOVA, $p<0.0004$). The number of new shoots/ node was similar for both types of modified MS (One-way ANOVA, $p=0.08$). Shoots produced on type A medium were on average twice as long as those from type B medium (One-way ANOVA, $p<0.02$) and there was no significant difference in propagation rate from either modified MS type A or B medium (One-way ANOVA, $p=0.31$). The percentage dry weight increase of shoots on modified MS medium type A was significantly higher than on the type B medium (One-way ANOVA, $p<0.0001$). The number of shoots with callus greater than 5mm in diameter was recorded at the end of the experiment and is expressed in table 4. 24. as a percentage of the total number of shoots in a particular treatment. A significantly higher percentage of shoots cultured on modified MS medium type A had callus compared to the type B medium (One-way ANOVA, $p<0.00001$) and it was noted that the callus growth on the type A medium was larger in size than that on the type B medium.

Viability levels were similar in both types of OM media tested. The number of new shoots produced per node was greatest for modified OM media type A but not significantly greater than for type B medium (One-way ANOVA, p=0.41). There was no significant difference in propagation rate from either type A or B medium (One-way ANOVA, p=0.49). Both the increase in dry weight of the shoots and the percentage of shoots with callus were similar for type A and B medium (One-way ANOVA, p=0.26 and p=0.14, respectively).

Table 4. 24. Viability (%), number of new shoots per node, mean shoot length (mm), propagation rate, dry weight increase (%) and percentage of shoots with callus greater than 5mm in diameter for explants of genotype 54 cultured on two types of modified MS medium and two types of modified OM medium.

	Modified MS		*Modified OM*	
	Type A	*Type B*	*Type A*	*Type B*
Viability (%)	100[a*]	50[b]	100[a]	100[a]
No. shoots/ node**	3.9±0.6[a]	2.1±0.4[a]	6.7±0.8[a]	5.8±0.7[a]
Mean shoot length **	4.4±0.6mm[a]	2.1±0.3mm[b]	3.0±0.5mm[a]	2.6±0.2mm[a]
Propagation rate**	2.9±0.6[a]	2.0±0.4[a]	3.3±0.7[a]	4.0±0.6[a]
DW*** increase (%)**	13238.6±1948.2[a]	2165.0±484.5[b]	7869.8±1296.2[a]	7237.1±1702.2[a]
Callus (%)	90.9[a]	10.0[b]	93.7[a]	75.0[a]

* Different letters between columns in the same media category i.e. between modified MS type A and B, indicate a significant difference according to Tukey's unequal N HSD at the 5% level. **± Standard error. *** Dry weight.

In a similar experiment, explants of genotype 49 were used to test the effect of culturing on modified OM medium type A and B. The results are shown in table 4. 25. Viability of the explants was similar for both media tested (One way ANOVA, p=0.42). Nodes cultured on modified OM media type A produced the greatest number of shoots but this was not significantly different to the amount produced on type B media (One-way ANOVA, p=0.28). There was no significant difference in the mean shoot length of the new shoots from either media tested (One-way ANOVA, p=0.55). The propagation rate was similar for both media tested (One-way ANOVA, p=0.37), as was the dry weight increase of shoots and the percentage of shoots with callus (One-way ANOVA, p=0.8 and p=0.79, respectively).

Table 4. 25. Viability (%), number of new shoots per node, mean shoot length (mm), propagation rate, dry weight increase (%) and percentage of shoots with callus greater then 5mm in diameter for explants of genotype 49 cultured on two types of modified OM medium.

	Modified OM	
	Type A	*Type B*
Viability (%)	91.7[a]*	83.3[a]
No. shoots/ node**	4.6±0.5[a]	3.8±0.5[a]
Mean shoot length (mm)**	3.1±0.2[a]	3.3±0.3[a]
Propagation rate**	4.0± 0.5[a]	3.3± 0.4[a]
Dry weight increase (%)**	1804.2± 285.8[a]	1531.7± 327.1[a]
Callus (%)	22.7[a]	26.6[a]

*Different letters between columns indicate a significant difference according to Tukey's unequal N HSD at the 5% level. ** ± Standard error.

4. 8. Discussion

The aim of the shoot production chapter was to identify the optimum culture regime, nutrient media formulation, carbohydrate source and growth regulator combination for micropropagation of healthy shoots of *F. excelsior* from a range of genotypes. Polyamine production was also monitored to provide an indicator of stress *in vitro*.

4. 8. 1. Culture regime

The results of this study have identified optimal culture regimes for shoot production of *F. excelsior.* Culturing shoots alternately on modified MS (M91) or OM medium containing growth regulators (BAP, TDZ and IBA) and QRC medium containing activated charcoal and no growth regulators provided the best shoot production (sections 4. 7. (2) and (4)). Culturing shoots continuously on medium containing growth regulators such as BAP and TDZ resulted in physiological disorders such as vitrification, shoot fasciation and excess callus production as well as increasing the occurrence of bacterial contamination (figures 4. 4 and 4. 11). In contrast, culturing shoots continuously on QRC medium with no growth regulators and activated charcoal gave low propagation rates due to reduced axillary branching (table 4. 5). By alternating the media, the explants were induced to produce axillary shoots on the growth regulator medium which were then transferred to medium with activated charcoal to elongate. This system produced healthy shoots, free from physiological disorders and reduced the opportunity for adventitious shoot production from callus, therefore, decreasing the risk of introducing somaclonal variation into the culture lines. Jona and Viglíocco (1985) added charcoal to medium on which *Prunus persica* shoots were to be elongated, finding that it overcame the negative effect of the high rate of cytokinin used in a shoot proliferation medium. *Quercus robur* is another species in which shoot elongation is reported to be

favoured by adding activated charcoal to the medium (Favre and Juncker, 1987). Exposure to rates of cytokinin that are too high can result in many small shoots which fail to elongate, unusual leaf shape and vitrification (George, 1993). TDZ in particular induces the production of callus and adventitious shoot organogenesis, which is not desirable in a clonal propagation system (Huetteman and Preece, 1993). Activated charcoal has been shown to absorb organic compounds in plant cell culture media including growth regulators (Fridborg and Eriksson, 1975).

4. 8. 2. Polyamines as indicators of plants stress in vitro

It was demonstrated that the culture regime during shoot production has an effect on the levels of polyamines being produced by the explants (section 4. 7. (1)). Shoots of *F. excelsior* cultured continuously on M91 medium (culture regime 2) had the highest concentration of all three polyamines measured (putrescine, spermidine and spermine) (figure 4. 22). Whereas, shoots cultured continuously on QRC medium with charcoal (culture regime 1) had the lowest levels of all three polyamines (figure 4. 21). Shoots in culture regime 3 (alternating between M91 medium and QRC medium with charcoal) had an intermediate concentration of polyamines between regime 1 and 2 (figure 4. 23). The concentration of polyamines fluctuated with each transfer in culture regime 3 for all genotypes tested, generally being higher after transfer to M91 medium and decreasing on QRC medium with activated charcoal. For some genotypes in culture regime 2, the concentration of putrescine fluctuated with each transfer, increasing initially after transfer to fresh medium then decreasing before the next transfer period. In all genotypes, putrescine was found in the highest concentration of all three polyamines followed by spermidine then spermine. Putrescine levels were lowest in culture regime 1 and were similarly higher for culture regimes 2 and 3 in all genotypes whereas, spermidine and spermine levels were higher in culture regime 2 compared to culture regimes 1 and 3. In culture regime 2 differences were recorded in spermidine concentration between genotypes, which did not exist in culture regime 3. Shoots of genotype 72 in culture regime 2 were suffering from vitrification, excess callus production and bacterial contamination by the end of the experiment and this was reflected in the putrescine content of the shoots which was among the highest measured during the course of the experiment. The variability in putrescine concentration measured between genotypes was particularly apparent in culture regime 3 (figure 4. 23).

The most dramatic changes in polyamine metabolism occur when plants are subjected to abiotic stress (Piqueras *et al.*, 2002). In this study, polyamine concentration, particularly that of putrescine, increased when the shoots were transferred from the low salt medium with activated charcoal (QRC) to the high salt medium containing plant growth regulators (M91). Tonon *et al.* (2004) used *Fraxinus angustifolia* callus cultures as a plant model to study the ionic and osmotic components of salt stress and their effects on polyamine metabolism. An early increase in putrescine was related to the combined osmotic and ionic stress and it was seen that the salt ionic component, whatever the stress intensity, interferes with the cells ability to accumulate spermidine and spermine in the

short term. It has often been suggested that putrescine and derived polyamines (spermidine and spermine) may have distinct functions in response to stress (Tonon *et al.*, 2004). In several plant systems (Drolet *et al.*, 1986; Bar *et al.*, 1996; Benavides *et al.*, 1997) it has been shown that spermidine and spermine act as anti-senescent compounds in response to stress, while putrescine produces either no effects or negative effects, such as depolarization of membranes, potassium leakage, tissue necrosis, and protein loss (Tiburcio *et al.*, 1990). In this context, Bouchereau *et al.* (1999) suggests that in response to both salt and osmotic stress, susceptible organisms would tend to accumulate putrescine and they would be unable to transform it in higher molecular weight polyamines (spermidine and spermine). By contrast, an increase in spermidine and spermine levels, generally observed in tolerant genotypes, would be an adaptive response to stress. In this study, genotype F5 accumulated the highest concentrations of spermidine and spermine (395.7 and 166.9 nmol g^{-1} FW, respectively) in culture regime 2 (continuous culture on M91 medium), compared to the other three genotypes tested. It was noted that shoot cultures of F5 in culture regime 2 were growing well and showed mild bacterial contamination. On the contrary, genotype S11 had the lowest concentration of both spermidine and spermine in culture regime 2 (87.9 and 33.7 nmol g^{-1} FW, respectively). It was noted that the S11 shoot cultures were not growing well and suffered from physiological disorders and bacterial contamination. These results indicate a greater capacity for stress tolerance in genotype F5 compared to the genotype S11. Furthermore, in genotype 49 a decrease in putrescine content was recorded from shoots transferred to M91 medium at the end of culture regime 3 (alternate culture on QRC with charcoal and M91 medium), this was associated with a similar decrease in propagation rate from 3 (day 210) to 1.4 (day 245). Tonon *et al.* (2004) noted that the long-term effects of salt stress resulted in growth inhibition and an associated decrease in putrescine levels in *Fraxinus angustifolia* callus cultures.

Elevation of polyamine levels in response to stress conditions has been well documented. For example, SO_2 fumigation, water stress, acidification, salinity, osmotic stress and cold exposure have been reported to increase polyamine levels, particularly putrescine, in plants (Priebe *et al.*, 1978; Kandpal and Rao, 1985; Shen *et al.*, 1994; Smith, 1985; Jouve *et al.*, 1995). In this study, the concentration of polyamines measured varied between genotypes within a culture regime indicating that the stress response was not uniform over all genotypes and variation in stress tolerance existed between genotypes. Variation in endogenous polyamine content between two different explants of the same system (Boget *et al.*, 1995) or different regions of a same explant (Altamura *et al.*, 1993; Sharma and Rajam, 1995) have also been observed in other studies and shown to be responsible for differential embryogenic and organogenic potential. Shoeb *et al.* (2001) studied the importance of cellular polyamine levels for plant regeneration in different genotypes of Indica rice (*Oryza sativa* L.) finding that plant regeneration ability in rice could be improved or even induced by regulating cellular PA pools. Transgenic lines of tobacco (*N. tabacum* var. *xanthi*) with increasing spermidine and putrescine titres, especially in the conjugated forms, exhibited varying tolerance to

salinity and drought as well as to fungal wilt caused by *Verticillium dahliae* and *Fusarium oxysporum* (Waie and Rajam, 2003).

Polyamines behave as polycationic and complexing agents within cells and this is probably the basis for their multiple functions; polyamines can affect membrane fluidity, act as free radical scavengers and control many aspects of DNA, RNA and protein turnover (Tiburcio *et al.*, 1993). Polyamines conjugated to phenolic compounds, hydroxycinnamic acid amides (HCAAs), have been shown to accumulate in incompatible interactions between plants and a variety of pathogens (Walters, 2003). The mechanism of action of polyamines in biotic stress reactions is not clearly understood, although polyamine conjugates have been shown to be antimicrobial in nature (Rajam, 1997; Martin-Tanguy, 1985). The physiological rationale for the correlation between abiotic stress and polyamine levels in plants is not known (Walters, 2003). However, work by Liu *et al.* (2000) showed that spermidine, spermine, cadaverine and putrescine strongly inhibited opening and closing of stomata in *Vicia faba*. Their work suggests that polyamines target inward potassium channels in guard cells and modulate stomatal movements, so providing a link with abiotic stress, polyamine levels and stomatal regulation. Several findings suggest that putrescine may have different effects than those reported for spermidine and spermine (Bouchereau *et al.*, 1999). Putrescine has been reported to cause depolarisation of membranes and increase potassium leakage (Tiburcio *et al.*, 1990). Spermidine and spermine have been shown to reduce ethylene synthesis and chlorophyll breakdown (Kushad and Dumbroff, 1991; Davies *et al.*, 1991). Spermidine and spermine might interact with membranes either by inhibiting transbilayer movement of phospholipdis, or by stabilizing molecular complexes of thylakoid membranes (Bratton, 1994; Popovic *et al.*, 1979; Besford *et al.*, 1993). Spermine, which has four amino groups, was a more effective scanvenger of superoxide than the triamine spermidine, suggesting the involvement of amino groups (Bouchereau *et al.*, 1999).

4. 8. 3. Culture medium

Modified Olive medium (OM) macro-, micronutrients and vitamins (Ruigini, 1984) have been identified as the optimum culture medium formulation for *in vitro* culture of *F. excelsior*. Explants from four different genotypes grew best on the modified OM medium compared to three other media formulations tested including modified MS, modified WPM and DKW (section 4. 7. (2.1)). The viability levels were high in all four media tested but were significantly reduced in one genotype cultured on modified MS medium. Furthermore, shoots from two genotypes were subjected to two culture regimes on media containing the modified MS and OM salts (section 4. 7. (3)), genotype S11 had similar propagation rates for both media and both culture regimes whereas genotype 49 grew best on modified OM medium. Further testing of modified OM and MS media was required due to an error in the concentration of certain micronutrients, but this only appeared to have significant effect for the modified MS medium (section 4. 7. (4)). Modified MS media type A (with nutrient levels as published by Murashige and Skoog (1962)) gave better results for all variables

measured compared to the type B medium (with altered nutrient levels). However, it was noted that a significantly larger proportion of explants had excessive callus growth in the type A MS medium (90.9%) compared to the type B medium (10.0%). Although callus production is frequently an essential step in the regeneration of adventitious organs, the growth of callus is believed to inhibit shoot proliferation in woody plant tissue culture (Hutteman and Preece, 1993).

Many species are propagated well in MS or slightly modified MS medium, and numerous authors agree that culture medium may not be a critical factor in culture growth (Ruigini, 1984). However, for *F. excelsior,* according to Hammatt (1994), the type of basal medium used during shoot production had the greatest effect on culture performance more than did the plant growth regulator concentration and furthermore, significantly affected the number of cultures that died, this being a particular problem with MS medium. It has been previously shown that forest trees grow better on media with weaker salt formulations (McCown and Sellmer, 1987) therefore, the high concentration of salts in MS medium may be a factor contributing to the poor growth of *F. excelsior* on this medium as shown in the results of this chapter (section 4. 7. (2. 1) and (3)). Shoot tip necrosis on MS medium has been reported for several other trees including *Pistacia* (Barghchi and Alderson, 1985) and *Dipterocarpus* (Linington, 1991). Most authors working with *F. excelsior* (Chalupa 1990; Hammatt and Ridout 1992; Hammatt 1994; Silveira and Cottignies 1994; Pierik and Sprenkels, 1997) and other *Fraxinus* species including *F. americana* (Preece *et al.,* 1987 and Preece *et al.,* 1989) and *F. angustifolia* (Perez-Parron *et al.,* 1994) observed the optimum culture performance on low salt media such as WPM and DKW which were both formulated specifically for the propagation of woody species (Llyod and McCown, 1980; Driver and Kuniyuki, 1984). Ruigini OM was successfully used to micropropagate *Olea europaea* (Ruigini, 1984), *F. ornus* (Arrillaga *et al.,* 1992) and more recently *Phillyrea latifolia* (Lucchesini and Mensuali-Sodi, 2004) an evergreen sclerophyllous species belonging to Oleaceae.

Of all four nutrient media tested in this chapter, the number of new shoots per node was highest on the modified WPM and OM medium (section 4. 7. (2. 1)), however, shoot length was lowest on modified WPM medium in three of the four genotypes tested. This suggests that the shoots need a low salt media to promote bud opening but a media richer in nutrients to support shoot elongation. Hammatt (1997) suspected that the poor performance of ash seedling cultures on WPM medium compared to DKW medium was due to reduced nutrition, especially nitrogen. Adding extra nitrogen in the form of NH_4NO_3 to the WPM medium resulted in less shoots but the shoots were larger and easier to handle. When Arrillaga *et al.* (1992) used Ruigini's OM to micropropagate *F. ornus* the use of a liquid induction medium of low salt concentration (Ruigini 1) favoured multiple shoot formation and effectively overcame the strong apical dominance of the species, but achieving the maximum shoot multiplication rate required an additional transfer to a version with a higher salt concentration (Ruigini 2).

Referring to the accidental increase in micronutrients in both the modified MS and OM media, George (1993) suggests that cell growth and morphogenesis of some species may be promoted by increasing the level of micronutrients above that recommended by Murashige and Skoog (1962). However, the need for macronutrient concentrations to be optimised as the first step in media development is emphasised by the results of Eeuwens (1976) who worked on growth and callus initiation of tissue explants excised from mature *Cocos nucifera* and cultured *in vitro*. In an experiment with factorial combinations of macro and micro-nutrient components of four media Eeuwens (1976) found that certain micronutrient formulations only gave improvements when combined with certain macronutrient formulations. This may explain why the increase in micronutrient concentration, in the experiments presented in this chapter, had a negative effect with the MS media but had no observable effect with the OM media.

4. 8. 4. Carbohydrate source

Experiments to determine the optimum carbohydrate source for *in vitro* culture of *F. excelsior* show the best viability rates are on media containing sucrose or mannitol (at 87.6mM) as the carbohydrate source and the highest propagation rates are on media containing either sucrose alone or in combination with mannitol (each sugar at 43.8mM). The results also varied depending on the genotype (section 4. 7. (2. 2)). Among the many available carbohydrates used in plant tissue culture, sucrose has been the major one in use and has been commonly used for *in vitro* propagation of *F. excelsior* (Chalupa, 1990; Hammatt and Ridout, 1992; Silveira and Cottignies, 1994; Pierik and Sprenkels, 1997), *F. angustifolia* (Perez-Parron *et al.,* 1994; Tonon *et al.,* 2001), *F. Americana* (Bates *et al.*, 1992), *F. ornus* (Arrillaga *et al.,* 1992) and *F. pennsylvanica* (Kim *et al.,* 1997). However, sucrose may cause hypoxia and ethanol accumulation in cells due to its quick metabolisation (Scott *et al.*, 1995; Ramarosandratana *et al.*, 2001). In some cases, sucrose is totally or partially replaced by other carbon sources (George, 1993; Rout *et al.*, 2000; Ramarosandratana *et al.*, 2001). Mannitol is a sugar alcohol that can be produced as a primary photosynthetic product and some species growing *in vivo* and *in vitro* can metabolise it (Trip *et al.,* 1964; Lipavska and Vreugdenhil, 1996). Mannitol occurs in most members of Oleaceae, where it comprises a significant proportion of the soluble carbohydrate pool (Stoop *et al* 1996; Peltier *et al.,* 1997). In tissue culture mannitol has been used as an osmotic agent (Leva and Muleo, 1993), used instead of sucrose in the *in vitro* culture of *O. europaea* (Leva *et al.,* 1994) and more recently for *in vitro* culture of *Phillyrea latifolia* (Lucchesini and Mensuali-Sodi, 2004). Sorbitol, another sugar alcohol, has been used for *in vitro* culture of *Pyrus pyrifolia* N. (Kadota *et al.,* 2001), *Prunus salicina* Lindl. (Harada and Muri, 1995) and crabapple (Albrecht, 1986). Harada and Muri (1995) found cultivar differences in *P. salicina* for sugar requirement and Albrecht (1986) reported similar findings for crabapple where some varieties grew best on sorbitol alone, some on sucrose alone and some requiring a mixture of sorbitol and sucrose. Another factor aside from the change in osmotic potential, which was ruled out in this study by using equimolar concentrations of the carbohydrates tested, is the changes that occur when the media is autoclaved with the different

carbohydrates. It has been previously shown that autoclaving culture media containing sucrose as the only carbon source may result in a 10-15% carbohydrate hydrolysis, which can cause a significant reduction in the osmotic potential of the media (George, 1993; Khuri and Moorby, 1995). Cultures of some plants grow better in media containing autoclaved rather than filter sterilised sucrose, suggesting that the cells benefit from the availability of glucose and fructose resulted from the autoclaving process (George, 1993). Owen *et al.* (1991) noted that media autoclaved with sucrose generally have a slightly lower pH than those autoclaved without it, but if maltose, glucose, or fructose has been added instead of sucrose, the post-autoclaved pH was significantly reduced.

4. 8. 5. Cytokinins

The optimum concentration of the cytokinins BAP and TDZ in the culture medium was similar for both genotypes tested. The highest propagation rate was from media with BAP (5mg/l) in combination with TDZ. Genotype 8 grew best on TDZ at a concentration of either 0.25 or 0.5mg/l where as genotype 49 grew best with 0.25mg/l TDZ in the medium (section 4. 7. (2. 3)). However, it was noted that the amount of callus growing on the nodes increased with increasing cytokinin concentration. Previous research on *Fraxinus* species has demonstrated that BAP, TDZ and IBA are effective plant growth regulators for axillary shoot proliferation (Navarrette *et al.*, 1989; Chalupa, 1990). High concentrations of BAP (1 – 5mg/l) stimulated shoot proliferation in juvenile and mature explants of *F. excelsior* (Chalupa, 1990; Leforestier *et al.*, 1990; Hammatt and Ridout, 1992; Hammatt, 1994; Silveira and Cottignies, 1994); *F. ornus* (Arrillaga *et al.,* 1992) and *F. angustifolia* (Perez-Parron *et al.,* 1994; Tonon *et al.,* 2001). Pierik and Sprenkels (1997) found that shoot length and multiplication rate of mature *F. excelsior* increased with increasing concentrations of the cytokinin tetra-hydropyranyl benzyladenine (PBA). Navarrete *et al.* (1989) observed that optimal axillary shoot proliferation and elongation in *F. americana* was stimulated by combining TDZ (3μM) with BAP (1μM) and IBA (1μM) in the culture medium. Kim *et al.* (1997) found the concentration of BAP and TDZ significantly affected shoot proliferation of *F. pennsylvanica*. Furthermore, it was observed that the numbers of "short shoots" suspected to of adventitious origin increased with increasing TDZ and BA concentrations. Compared to most other compounds with cytokinin activity, lower concentrations of TDZ can stimulate axillary shoot formation in many woody plants, where as higher concentrations of TDZ may result in the formation of both axillary and adventitious shoots (Chalupa, 1988; Van Nieuwkerk *et al.,* 1986). High rates of shoot proliferation, often desirable for efficient micropropagation, may include both axillary and adventitious shoots. If clonal fidelity is required, however, TDZ or other cytokinins must be used at levels that stimulate only axillary shoot growth, thereby avoiding potential somaclonal variants derived from adventitious shoots. Bates *et al.* (1992) used TDZ to promote adventitious shoot formation from *F. americana* callus, neither BA nor 2ip stimulated formation of adventitious structures in this study. Adventitious regeneration was stimulated in *F. excelsior* by TDZ from hypocotyls, cotyledons (Tabrett and Hammatt, 1992) and whole leaves (Hammatt, 1994).

4. 8. 6. Genotypic and physiological factors affecting shoot proliferation

The results in this chapter illustrate that substantial variation exists between and within genotypes of *F. excelsior* with respect to their ability to grow and proliferate on the tissue culture medium. This may relate to the physiological state of the shoots at the time of experimentation or to the genetic variation among the genotypes tested. For example, propagation rates for genotype F5 cultured alternately on M91 and QRC medium with charcoal (table 4. 5) varied from 1.5 to 3.0 (for the shoots sub-cultured off QRC medium with charcoal only). In this case the higher propagation rates were associated with a decrease in putrescine concentration (figure 4. 8) indicating that the shoots were at different physiological states during the course of the culture regime and this had an effect on the propagation rate. The possible role of polyamines as regulators of physiological processes in plants has been described by numerous authors (Galston and Kuar-Sawhney, 1987; Bagni *et al.*, 1983; Altman *et al.*, 1977, Hiatt, 1989; Flores, 1990). The results of Shoeb *et al.* (2001) showed that regeneration potential in *Oryza sativa* could be altered by modulation of the cellular polyamine pools. Galston and Kaur-Sawhey (1990) showed that the application of exogenous polyamines to intact plants or plant parts produced visible effects such as prevention of senescence in excised leaves and the formation of somatic embryos or floral primordia in otherwise vegetative tissue cultures.

As demonstrated in this chapter and during the initiation experiments in chapter 3, different genotypes of *F. excelsior* can display genotypic differences for their *in vitro* growth requirements, perhaps indicating differences in their ability to use nutrients. Nas and Read (2004) observed similar findings for individual genotypes of *Corylus avellana* L. and suggested the need to dilute the culture medium to suit the requirement of each genotype. They concluded that the effect of genotype cannot be underestimated and the use of the right culture medium seems to be the key factor determining successful micropropagation of a large number of genotypes. However, on a commercial scale it is not feasible to develop individual media concentrations for each genotype in culture. The study in this chapter has shown that a range of genotypes of *F. excelsior* can be micropropagated successfully by culturing alternatively on modified OM medium supplemented with BAP (5m/l), TDZ (0.55mg/l), IBA (2mg/l), and QRC medium with activated charcoal using sucrose as a carbohydrate source. This system delivers a good rate of shoot production with healthy shoots, free from physiological disorders and reduces the risk of introducing somaclonal variation into the culture lines. The polyamine production from ash shoots in three different culturing regimes was revealed as an indicator of plant stress *in vitro* and the concentration of spermidine and spermine was observed to be highest in a genotype better adapted to stress compared to a genotype suffering from stress as indicated by physiological disorders such as vitrification and spindly leaves. Once again, it was seen that the *in vitro* response of *F. excelsior* shoots depends on the genotype of the stock plant and that a high level of variation exists with respect to the ability of plants to grow on the tissue culture medium.

CHAPTER 5: Root Induction and Plant Growth

5. 1. Introduction

The rooting of shoots produced *in vitro* is an important part of any propagation scheme. Therefore, it is necessary to find a method of root induction for *F. excelsior* that is both reproducible and suited for a range of genotypes. The ability of plant tissues to form adventitious roots depends on interactions of many different endogenous and exogenous factors. Auxins are the main factors involved in root formation (Németh, 1986). However, not all root primordia initiated after auxin treatment develop into emergent roots (Mitsuhashi-Kato *et al.*, 1978) and for root elongation exogenous auxin is usually not required or is even inhibitory (Torrey, 1956; Mohammed and Eriksen, 1974; Mitsuhashi-Kato *et al.*, 1978). Determination of media and environmental conditions to promote adventitious root induction and elongation is important.

Adventitious roots are defined as those originating in locations other than the primary root system (Blakesley *et al.*, 1991a). Consequently a cutting from the parent plant, may under certain conditions produce adventitious roots and hence a new root system and a complete plant. In spite of the differing origin of the two types of root (primary roots and adventitious roots), their physiological functions are essentially the same (Blakesley *et al.*, 1991a). Whilst acting as supports, they both offer a means of extracting water and nutrients from their surroundings. However, it is important to make a distinction between adventitious root primordia that have been induced *de novo* and those that are preformed. Preformed root initials are formed in the intact plant in the normal development of that plant (Haissig, 1974). They are usually slow to develop, are found almost exclusively in woody plants and occasionally become evident externally as overt or aerial roots. The site of formation of preformed root primordia varies between species, although they have frequently been associated with ray tissue (*Thuga* species) and nodal regions (Malvaceae) (Fink, 1972; Campen *et al.*, 1990). Anatomical studies on a wide range of plant material have shown that adventitious root formation usually occurs in the vicinity of differentiating vascular tissues (Hartmann and Kester, 1975), but within this generalisation there are many sites of origin. Similarly to preformed root primordia, these sites are rays associated with phloem and cambium, or in bud or leaf gaps (Blakesley *et al.*, 1991a). Although induced root primordia are found in internodal as well as nodal regions, they may also occur in callus tissue produced at a wound surface (Blakesley *et al.*, 1991a). Microscopic analysis of adventitious roots formed on *F. excelsior* shoots *in vitro*, showed a direct connection between the vascular elements of the adventitious roots and the stem of the plantlet (Silveira and Cottignies, 1994).

5. 2. Adventitious root development

Adventitious rooting can be divided in three phases: induction, initiation and expression (Kevers *et al.*, 1997); in practical terms, the last two phases may be condensed under the denomination of root formation phase (Fett-Neto *et al.*, 2001). Several workers have attempted to define distinct stages in adventitious root formation (Doré, 1965; Girouard, 1967; Smith and Thorpe, 1975), all of which essentially include the following (taken from Blakesley *et al*, 1991a):

(1) Dedifferentiation and the formation of a new meristematic locus: this is the primary event of adventitious root initiation.

(2) Early cell divisions: these produce a cluster of cells showing no polarity and are essentially radially symmetrical. At this stage the cells may not yet be determined in their new developmental pathway.

(3) Later cell divisions form an organised determined root meristem: it has been reported that the minimum size for a determined root meristem is 1500 cells (Torrey, 1963; White and Lovell, 1984). This is characterised by its bilateral symmetry.

(4) Formation of the root by extension growth of cells produced by the meristem: this stage includes the formation of the vascular union between the new meristem and the stem, and is the stage at which the root first becomes visible externally.

5. 2. 1. Auxin and root induction

The notion that substances which promote rooting are present in leaves and that they are transported to the base of the cutting, originated towards the end of the last century (Sachs, 1880). It became more popular following the work of van der Lek (1925), who demonstrated that active buds produced a substance(s) which promoted rooting, since rooting was inhibited after an incision was made through the vascular system between the bud and the site of root initiation. Known sites of auxin production, including leaves and buds, have been found necessary for root initiation in a number of species (Blakesley *et al.*, 1991a). Replacement of buds and/ or leaves by added auxin has been partly or completely successful in stimulating adventitious rooting in Douglas Fir, *Pisum* and *Salix fragilis* (Blakesley *et al.*, 1991a). Furthermore, auxin movement is preferentially basipetal demonstrated in earlier work by Eliasson (1972) on *Populus tremula* and later by Marks (1996) in experiments with *Betula pendula*, *Daphne cneorum* and *Quercus robur*.

There is considerable evidence that auxin is not needed during all stages of adventitious root production. Indeed some evidence suggests an inhibitory action at a later stage. Nahlawi and Howard (1973) found that plum rootstock cuttings rooted better with an initial application of IBA, than with repeated applications. Furthermore, leafy pea stem cuttings were shown to be sensitive to auxin only during the first three days after the cutting was taken (Mohammed and Eriksen, 1974). Measurement of the endogenous levels of IAA in the rooting zone of cuttings should provide more direct evidence and indicate how changing auxin levels control or influence the different phases of root initiation. Several workers have found a peak in the level of IAA in the rooting zone associated with the first stage of root initiation (Blakesley, 1984; Maldiney *et al.*, 1986; Moncousin *et al.*, 1988). In each case the level of IAA subsequently declined. There are other instances where no increase in IAA was observed, only a gradual decline (Berthon *et al.*, 1989; Blakesley *et al.*, 1991b).

Auxin is transported basipetally in the shoot, consequently when a cutting is excised, auxin might be expected to accumulate at the base. Several reports show that this accumulation is aided by reduced activity of IAA oxidase/ peroxidase in the rooting zone (Berthon *et al.*, 1989; Chibbar *et al*, 1979; Moncousin *et al.*, 1988). Vine cuttings show a transitory decrease in peroxidase after excision and this has been shown to coincide with an increase in IAA concentration (Moncousin *et al.*, 1988). The IAA concentration subsequently declined, accompanied by increased peroxidase activity. Jarvis (1986) proposed a model in which auxin accumulation is associated with the formation of the meristematic locus and the early cell divisions of the

second phase. The third phase (continued cell divisions to form an organised meristem) is associated with low levels of IAA. Severance of a shoot from its root system will inevitably have considerable effects on movement and localised accumulation of assimilates and plant growth substances. An intact plant is considered to be transporting cytokinin from the root to the shoot. Excision of the shoot removes this source of cytokinin and might, therefore be expected to lead to a reduced cytokinin concentration at the base of the shoot (Blakesley *et al.*, 1991a). Preliminary studies of cytokinin levels in cuttings of *Cotinus* and *Phaseolus aureus* showed that whilst cytokinins were easily detectable at the time of excision, their concentration fell dramatically to very low levels on subsequent harvest times (Blakesley, 1984). When cuttings are excised, cells at the base are likely to be exposed to changing ratios of auxin to cytokinin which might stimulate the start of the events of the cell cycle (Blakesley *et al.*, 1991a).

Auxin treatments are a common practice to induce rooting in reluctant species as well as to promote rooting (by increasing the number of roots and hastening their formation) in spontaneous rooting species (Kracke and Cristoferi, 1983; Davies, 1984; Eliasson and Areblad, 1984; Alvarez *et al*, 1989). Synthetic auxins such as IBA and NAA, have often been shown to be more effective than naturally occurring IAA, probably due to the faster metabolism of IAA compared to their synthetic analogues in most species (Jarvis and Booth, 1981; Nordstrom and Eliasson, 1984; Hammerschlag *et al.*, 1987). Chalupa (1990), Hammatt and Ridout (1992) and Hammatt (1994) report successful root induction *in vitro* with *F. excelsior* using IBA.

5. 2. 2. The influence of light on rooting in vitro

Hartmann *et al.* (1990) reports that some plants root best if stored in darkness during the auxin treatment phase. Boxus and Quoirin (1974) mentioned that light was inhibitory for rooting shoots of *Prunus*. Hammerschlag (1982) increased rooting percentages in *Prunus* by keeping shoots in darkness during the first two weeks of root induction then transferring them to illumination. Sriskandarajah *et al.* (1982) obtained high frequency of rooting in *Malus* under continuous illumination, whereas Welander (1983) found increased rooting of *Malus* shoots kept in darkness. Inhibition of rooting, an increase in callus and deterioration of the shoot can occur when explant material is exposed to auxins over extended periods (George, 1996). Exposure to light is sometimes thought to degrade the auxin and enhance root initiation and growth (Fabijan *et al.*, 1981). In many plants, the induction of roots is promoted by darkness or light of low fluence, apparently because natural auxin levels increase in these conditions, whereas they are decreased by light of high fluence (George, 1996). Alternatively, shoot material can be placed on a medium high in auxins for a short period (usually several days until root induction takes place) and then transferred to an auxin free medium to allow roots to develop and grow (George, 1996). Incorporation of activated charcoal (AC) is often used to neutralize any excess auxins that may be carried over after the initial pulse phase with auxin.

5. 2. 3. The use of cytokinins for root induction in vitro.

Many studies report the complete omission of cytokinins for promoting *in vitro* root development, in fact it is generally thought that high concentrations of cytokinin (0.5-10mg/l) inhibit or delay root formation (Harris and Hart, 1964; Ben-Jaacov *et al*, 1991) and also prevent root growth and the promotive effects of auxins on root initiation (Humphries, 1960). Despite these observations, there are reports that cytokinins can sometimes induce or promote root growth (Fries, 1960), or adventitious root formation, even in the absence of auxins

(Nemeth, 1979). In nearly all cases low levels of cytokinin have been effective, for example, shoots of sugar beet were rooted on MS medium containing 0.5mg/l kinetin and no auxin (Konwar and Coutts, 1990). Boxus and Terzi (1988) advocated the addition of 0.5mg/l kinetin and auxin to the rooting medium for strawberries and several woody plants, finding that at this concentration, the cytokinin had a bacteriostatic effect and rooting was not impaired. Johnson (1996) comments on the enhancement of rooting in *Eremophila lanii* taking place in the presence of 1μM of either cytokinin, kinetin or BAP, rather than an increase in auxin levels. These observations suggest that an optimal endogenous auxin-cytokinin balance might have been established for root formation. Although, cytokinins are considered to inhibit rooting, at later stages, the inhibitory effect of cytokinins disappear and development of root primordial seems to depend on cytokinins (Eriksen, 1974).

Some studies have suggested that a cytokinin carry over from the shoot multiplication phase is enough to suppress root development. Removal or "leaching" of excess cytokinin from shoots is often critical since they generally antagonize rooting (Krikorian, 1995). The removal of excess cytokinin from the micro-cuttings can be carried out on a "blank" medium prior to exposure to auxin (Krikorian, 1988). Plants known to be hard-to-root because of endogenous inhibitors can profit by transfer to a medium that removes or destroys the inhibitor(s). Sometimes transfer to a medium containing activated charcoal helps to remove excess hormone (and other inhibitors) and thus promote root induction (George, 1993; Krikorian, 1995).

5. 2. 4. Other factors influencing adventitious formation in vitro

Roots have cells especially adapted for nutrient uptake and therefore take up salts more efficiently than other organs, or unorganized tissues, and grow well in a medium of low total salt concentration (George, 1993). Dilute media are also found to be most suitable for root initiation on isolated shoots (George, 1993). Bunn *et al.* (1989) found that shoots of a difficult to root Australian species, *Leucopogon obtectus*, formed roots on a minimal medium containing only agar and water. Studies involving many *Fraxinus* species including *F. excelsior* show that optimal rooting is achieved on the low salt WPM medium (Preece *et al.*, 1987, Chalupa, 1990; Hammatt and Ridout, 1992; Silveira and Cottignies, 1994 and Perez-Parron *et al.*, 1994).

In vitro root induction can also be improved by adding polyamines, in particular putrescine to the root induction media (Ruigini *et al.*, 1993). Inhibitors of polyamine synthesis have been found to prevent the formation of roots by thin layers excised from the floral stems of tobacco (Tiburcio *et al.*, 1989; Torrigiani *et al.*, 1990). A series of works on rooting of poplar shoots showed an early typical elevation of putrescine, close to the IAA peak, and to termination of the inductive phase (Hausman *et al.*, 1994, 1995). In these studies, the role of putrescine in the root induction phase was implied because the transient elevation of putrescine did not occur in non-rooting cuttings, putrescine was only measurable in the basal rooting zone. Inhibitors of putrescine biosynthesis applied prior to or at the beginning of the inductive phase inhibited rooting. An inhibitor of putrescine conversion to spermidine and spermine which promoted the accumulation of endogenous putrescine favoured rooting in the absence of exogenously applied auxin and finally, exogenously applied putrescine, prior to or at the beginning of the inductive phase, had a positive effect on rooting. Exogenously applied putrescine has been proven to increase rooting in apple (M9 rootstock) and olive but had no effect on chestnut, almond, jojoba and apricot and decreased rooting in walnut (Ruigini,

1993). It is hypothesized that IAA and putrescine, which are known to control cell division cycles (Del Duca and Serafini-Fracassini, 1993), are required to initiate cell divisions at the end of the inductive phase. However, the considerable peroxidase variation that occurs in the same time period may indicate a possible role of peroxidase in the relationship between IAA and putrescine. Gaspar and Hausman (2002) formulated the following hypothesis regarding the roles of peroxidases in the interplay between IAA and polyamines:

(1) Putrescine can inactivate some peroxidases involved in auxin catabolism by providing H_2O_2 through its catabolic effects, thus favouring an increase in the level of endogenous IAA.

(2) An increase in putrescine level might enhance peroxidase activity and hence bring about a decrease in IAA level.

Rooting in some ways might be considered a response to stress e.g., wounding due to cutting and an imposed hormonal imbalance in favour of auxin (Gaspar and Hausman, 2002). Indeed, a γ-aminobutyric acid (GABA) shunt of tricarboxylic acid cycle, from glutamate (Shelp *et al.*, 1995) or from polyamines (Bisbis *et al.*, 1997) to succinate, has been shown to operate under certain stress conditions. Succinate is a precursor for the biosynthesis of tetrapyrrole-containing compounds such as peroxidases via the Shemin pathway (Castelfranco and Beale, 1983).

Rooting success has been shown to improve in some plants following the addition of *Agrobacterium rhizogenes*. *Agrobacterium rhizogenes* is a soil gram-negative bacterium that induces adventitious root formation at a site of infection in a large number of plants (Chilton *et al.*, 1982). Several authors report successful rooting using *A. rhizogenes*-meditated transformation in fruit trees such as almond (Damiano *et al.*, 1995), apple (Sutter and Luza, 1993) Kiwi (Ruigini *et al.*, 1991) and walnut (Caboni *et al*, 1996). Root induction is due to the integration and subsequent expression of a portion of bacterial DNA (T-DNA) from the root inducing (Ri) plasmid in the plant genome (Damiano and Monticelli, 1998). Four loci involved in the root formation were identified in the T-DNA, and called root loci (*rol*) A, B, C, D (Spena *et al.*, 1987). The first step in the infection process is the host/pathogen interaction, where several factors contribute to the establishment of the relationship (Winans, 1992). Sugars and phenolic compounds released from the plants and membrane-binding protein can differ according to plant genotype, thus producing different responses. McAfee *et al.* (1993) proposed a role for the bacterium in modifying the root environment by means of organic acid secretions and lowering the pH.

Since the development of techniques in molecular biology, the identification of genes expressed in plant tissues is becoming possible using mutants (Aeschbacher *et al.*, 1994) and international data banks of genes already sequenced in various species have been set up. Up to recently, very few genes were identified specific to adventitious root development. Several major genes have been cloned and sequenced from roots at different developmental stages, but often their function remains unknown (Duroux *et al.*, 1998). However, these are suitable markers to be used for accurate characterisation of the different root developmental stages and good candidates to study their potential role in rhizogenesis through genetic transformation (over- or under-expression of a gene using RNA sense or antisense) and *in situ* hybridisation (histolocalisation of specific mRNA). The screening of genes specifically expressed in cells forming adventitious root meristems could be used to identify new factors controlling processes through specific

cellular mechanisms (Duroux *et al.*, 1998). Sitbon and Perrot-Recchenmann (1997) reported interesting results concerning the up- or down-regulation of genes by auxin that could be involved in the rhizogenic process. Among the auxin up-regulated genes, some of them including *hrgp-nt3* and *rsi-1,* are root-specific and expressed in a very early stage of root primordium formation (Taylor and Scheuring, 1994; Vera *et al.*, 1994). In both cases, their expression was investigated *in planta* respectively either by *in situ* hybridisation or by expression of the *gus* gene fused with the promoter of the corresponding gene.

5. 3. Weaning plants to the *ex vitro* environment

The acclimatization of micropropagated plantlets is a critical stage in micropropagation (Huylenbroeck and De Riek, 1995). In the first few weeks after transfer to *ex vitro* conditions plants cope with different stresses and have to adapt to the new environmental conditions (Huylenbroeck *et al*, 1995; Preece and Sutter, 1991). Substantial numbers of micropropagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environments (Preece and Sutter, 1991). The greenhouse and field have substantially lower relative humidities, higher light level and septic environments that are stressful to micropropagated plants compared to *in vitro* conditions (Preece and Sutter, 1991).

Acclimation is not unique to micropropagation and has been used in conventional propagation for years. Examples of two common techniques to acclimatise or wean plants are to gradually reduce the relative humidity or amount of mist (Hartmann *et al.*, 1990) and to maintain light level in the greenhouse at 50% shade before plants are transplanted to the field (Welch, 1970). Both of these techniques have been successfully used to wean tissue culture produced plantlets of *F. excelsior* and other *Fraxinus* species (Chalupa, 1990; Arrillaga *et al.*, 1992; Silveira and Cottignies, 1994 and Perez-Parron *et al.*, 1994). Rooted cuttings that have not been properly acclimatised frequently show signs of stress including wilting, marginal and tip necrosis of the leaves, and death of entire leaves or plants (Preece and Sutter, 1991). Plantlets or shoots that have been rooted *in vitro* are similar to cuttings rooted under mist since they have been exposed to conditions providing significantly less water stress than plants grown in the greenhouse or field. Shoots and plants in culture are grown in conditions that provide little physiological stress since a carbon source (typically sucrose) is provided, reducing the need for photosynthesis (Preece and Sutter, 1991). Shoots developed in culture have often been produced in high humidity and low light intensity. Resulting in there being less leaf epicuticular wax than on plants raised in growth chambers or the glasshouse (George, 1993). In some plants, the stomata of leaves produced *in vitro* may also be atypical and incapable of complete closure under conditions of low relative humidity, resulting in rapid water loss when tissue cultured plants are moved to external conditions (Sutter and Langens, 1979, 1980). Physiological changes including a switch from a hetertrophic or mixotrophic metabolism to autotrophism and morphological changes in stomatal behaviour and cuticular wax composition are necessary for the plants survival *ex vitro* (Capellades *et al.*, 1990; Donnelly *et al.*, 1985; Ziv, 1991).

Although most micropropagation literature still describes rooting *in vitro* there is an increasing interest, especially in commercial operations for rooting *ex vitro* (Debergh and Read, 1991). Several authors working with *Fraxinus* species report successful *ex vitro* rooting of shoots produced by micropropagation (Bates *et al.,* 1992; Kim *et al.,* 1997). However, depending on the species, rootless micro-shoots may die in large

numbers when removed from culture, but if losses are low then rooting and acclimisation can be done simultaneously (Preece and Sutter, 1991). This can substantially reduce costs of micropropagation and add efficiency to production schemes, and has been shown to be effective in many woody species including walnut, peach and apple (Driver and Suttle, 1987; Welander, 1983; Zimmerman and Fordham, 1985).

Chalupa (1990) report that weaned *F. excelsior* trees planted in the field after two months hardening in the greenhouse survived well over winter and grew normally with good clonal uniformity. Endodormancy in temperate-zone deciduous trees is a phase of development that allows the trees to survive unfavourable conditions during the winter (see chapter 2), a hallmark of which is that it is released by a quantitative accumulation of a certain amount of winter cold (Faust *et al.*, 1997). Plants may enter this stage of dormancy independently of the environmental conditions but are incapable of emerging from it autonomously (Saure, 1985). The symptoms of prolonged dormancy described by Chandler *et al* (1937), Hill and Campbell (1949), Black (1952), and Skinner (1964) include: delayed, protracted and very weak leafing; formation of bare unbranched shoots that become increasingly shorter due to shorter internodes; shortage of spurs capable of forming flower buds; quickly declining growth vigour and, thus, early senescence of the trees and a delayed and protracted flowering season followed by poor fruit development and irregular ripening. Therefore, it may be important to give weaned plants chilling over winter to ensure normal growth resumes in the spring.

5. 4. Rooting stem cuttings from mature trees

The increased knowledge of the effects of juvenility on stock plants used to produce cuttings for rooting, has been one of the most important factors in improvements of propagation technology (Kunneman, 1995). The adventitious root initiation potential of stem tissue of some species (particularly tree species) may decrease during ontogenetic aging and maturation. Cuttings from young trees and shrubs usually propagate easily from stem cuttings, but by the time they have been tested and selected for desirable characteristics, they lose much of their potential to root (Hackett, 1985). For these reasons, the ability to obtain relatively juvenile material from mature plants is very desirable. The results of many studies indicate that phase- related characteristics can be modified through *in vitro* culture and both the length of culture and the number of sub cultures involved seem to be related to such changes (Hackett, 1985; Hammatt, 1994, 1997; Pierik and Sprenkels, 1997).

Many authors measure the level of rejuvenation *in vitro* in terms of increased rooting potential (Gupta *et al.*, 1981; Lyrene, 1981; Sriskandarajah *et al.*, 1982). Tissue culture is now commonly used to rejuvenate stock plants and improves the rooting of many taxa (Kunneman, 1995). Douglas (personal communication) observed that cuttings from *F. excelsior* trees produced by micropropagation techniques, and taken during the actively growing period had good rates of root production. The use of leafless hardwood cuttings is a traditional propagation method for easy-to-root woody plants (Schmit, 1995). Hardwood cuttings of some problematic species root as well or even better, than leafy softwood cuttings if special techniques are used such as providing bottom heat and rooting cuttings indoors under a plastic tunnel in a heated greenhouse (Howard, 1968; DeBoer and Van Elk, 1983; Joustra and Verhoeven, 1984; Howard and Harrison-Murray, 1988; MacDonald, 1989; Schmidt, 1995). The advantages of using hardwood cuttings in a propagation

system include the extension of the propagation season by utilising the relatively free months of late winter; shortening production time and giving the plants an earlier start to growth.

The rooting phases have specific requirements and the recognition of their features is important for the adequate manipulation of this developmental event. Many environmental and endogenous factors regulate rooting (Hartmann *et al.*, 1990; Blakesley *et al.*, 1991b). Phytohormones play complex roles, exerting direct (acting on cell division or cell growth) or indirect (interacting with other molecules or phytohormones) effects (Eriksen, 1974; Blakesley *et al.*, 1991b).

The aim of the experimental work in this chapter was to elucidate a reproducible and efficient way to produce rooted plantlets from the shoot production stage. This was done by manipulating auxin concentration and auxin exposure time, experimenting with darkness to promote rooting and testing different salt concentrations and the addition of BAP in the rooting media. Following the rooting phase the plantlets were weaned to the glasshouse and their progress monitored. An experiment was set up to test the rooting potential of dormant winter cuttings from mature trees vegetatively propagated through the micropropagation cycle and grown in the greenhouse compared to those propagated by grafting and grown in the field.

5. 5. Materials and methods

A. Plant material

Shoots for the root induction experiments were harvested from well established cultures (in the culture cycle for at least 18 months) derived from mature trees of selected genotypes 47, 49, 70 and non-selected genotype F5 growing as grafted plants in the field and/ or greenhouse at Teagasc, Kinsealy (refer to chapter 3 section 3. 7. A (2)). Shoots were also used from cultures of genotypes S8 and S11 derived from commercial seed. (For a full description of culture media and establishment protocol refer to chapter 4 section 4. 6. B and C). It must be noted that the shoot cultures used in the following experiments were not derived from the initiation experiments described in Chapter 3.

B. Shoot cultures used for rooting experiments

The shoots used in rooting experiments were collected from cultures routinely subcultured by transferring the cultures alternately from M91 medium containing MS based salts and plant growth regulators, to QRC medium, a low salts medium, supplemented with activated charcoal (described in chapter 4 section 4. 6. C). Shoots were available from different cultures at different times and were used accordingly.

C. Root induction.

The explants used in rooting experiments were collected from QRC media with charcoal except where stated otherwise. The explants used for experimentation consisted of a shoot apex and one or two nodes except where stated otherwise, alternatively in one experiment explants with the shoot apex removed and containing one node with a section of internode below were used (section 5. 5. F. (1)). Both shoots types used in root induction experiments were approximately 1-2cm in length. In most cases rooting was carried out on 6mls of medium solidified with 8.5g/l agar and containing 20g/l sucrose as a carbohydrate source, in

50mm Petri dishes, sealed with Parafilm (two shoots per dish), unless stated otherwise. In other cases, 200ml powder jars with screw top lids (two shoots per jar) and containing 30mls of medium were used. The Petri dishes (or jars) in each experiment were arranged randomly on the same shelf in the growth room. Cultures were kept in the growth room at 22°C with a 16-hour photoperiod under Philips cool white fluorescent tubes (75 µmol m^{-2} s^{-1} at bench level).

D. Weaning

After each root induction experiment, rooted or non-rooted plantlets were transferred to the greenhouse in seed trays containing a mixture of peat and perlite (1:1 v:v), the seed trays were covered with plastic propagator lids and the vents on the lids closed. After approximately two - three weeks the lid vents were opened, and after a further four weeks the propagator lids were removed. The temperature in the greenhouse was regulated by heating in the winter and ranged from 15-22°C, artificial lighting was also provided in winter by Philips SON 400W lights (16.5 µmol m^{-2} s^{-1} at bench level) with a 16-hour photoperiod.

E. Data collection and analysis.

Root induction experiments lasted for five weeks (unless stated otherwise). Any contaminated material was discarded from experiments. At the end of each experiment recordings were made of the presence or absence of roots, root number and root length.

Statistical analysis was performed using Statistica 6.0 statistical package. The percentage of rooted shoots were analysed using a Chi square test, and arc sin transformed (to normalise the data) for analysis by factorial ANOVA. Root counts were square root transformed and analysed by ANOVA. Root length data were analysed by ANOVA. Mean separation was performed using Tukey's honest significant difference (HSD) test. Data on the survival rate of weaned shoots was analysed by a two-sided Students T-test designed to test for differences between two proportions. Significance was recorded at $p \leq 0.05$. All descriptive statistics reported in text and tables is from the non-transformed data, even if the statistical tests were done on the transformed variable.

F. Experimental treatments for root induction

(1) The effect of continuous exposure to different IBA concentrations on root induction.

Apical and nodal explants were collected from genotypes S11 and 49. Shoot tips from each genotype were placed in Petri dishes containing QRC medium (chapter 3 section 3. 7. B) with 0, 5, 10, 15 and 20mg/l IBA. Each treatment had 12 replicates with two individuals per replicate. Nodes from each genotype were placed in Petri dishes containing QRC medium with 0, 10 and 20mg/l IBA. Each treatment had 10 replicates per genotype with two individuals per replicate.

In this experiment it was noted that a number of explants had substantial callus growth on the stem base, in order to quantify this variable, the diameter of the callus base on each shoot was measured and the percentage of shoots with callus greater that 5mm in diameter was recorded.

(2) The effect of a dark period of 0, 2, 4, 8 and 10 days during continuous exposure to IBA on root induction.

Shoot tips were collected from genotypes S11 and 49. Apical shoots from each genotype were placed in Petri dishes containing QRC medium with 10mg/l IBA. The dishes were wrapped in aluminium foil to exclude any light, after 0, 2, 4, 8 or 10 days groups of 10 dishes were removed from the aluminium foil and placed in the light. Each treatment had 10 replicates per genotype with two individuals per replicate.

(3) The effect of a dark period and pulse exposure for 0, 2, 4 and 8 days to IBA on root induction.

Shoot tips were collected from genotype F5 and placed on QRC medium containing 1mg/l IBA. The shoots were kept in dark or light conditions for 0, 2, 4 or 8 days on the IBA medium before being transferred to QRC medium with charcoal in the light. Each treatment had 12 replicates with two individuals per replicate.

(4) The effect of pulse treatment of two days versus continuous exposure to IBA at different concentrations on root induction.

Shoot tips were collected from genotypes S8 and 47. Apical shoots from each genotype were placed in Petri dishes containing QRC medium with 0, 10 or 20mg/l IBA. Explants in the pulse treatment were removed from the IBA medium after two days and transferred to QRC medium with charcoal. Each treatment had 10 replicates per genotype with two individuals per replicate.

In this experiment it was noted that a number of explants had substantial callus growth on the stem base, in order to quantify this variable, the diameter of the callus base on each shoot was measured and the percentage of shoots with callus greater that 5mm in diameter was recorded.

(5) The effect of a pulse exposure for two days at different IBA concentrations on root induction.

To further test the effect of IBA concentration on rooting during a pulse exposure treatment, shoot tips were collected from genotypes S11 and 47. Shoots from each genotype were placed in Petri dishes containing QRC medium with 0, 5, 10 or 20mg/l IBA. The Petri dishes were wrapped in aluminium foil to exclude any light and after two days were removed from the IBA medium and transferred to QRC medium with charcoal in the light. Each treatment had 12 replicates per genotype with two individuals per replicate.

(6) The effect of a pulse treatment for two days on 10mg/l IBA in darkness on root induction in two genotypes.

Shoot tips were collected from genotypes F5 and 70. Shoots from each genotype were placed in Petri dishes containing QRC medium with 10mg/l IBA. The Petri dishes were wrapped in aluminium foil to exclude any light and after two days were removed from the IBA medium and transferred to QRC medium with charcoal in the light. Each treatment had 12 replicates per genotype with two individuals per replicate.

(7) The effect of three media darkening methods during continuous exposure to IBA on root induction.

Shoot tips were collected from genotypes S8 and 49. Shoots were placed on QRC media with 10mg/l IBA in jars. The media was darkened using three methods: 1) wrapping the base of each jar in aluminium foil and covering the top of the media with a sterile section of aluminium foil, 2) adding 100mg/l of the dye Brilliant Black BN (SIGMA B8384; C.I. 28440) or 3) adding 3g/l of activated charcoal. The control had no media darkening. Each treatment had 12 replicates per genotype with two individuals per replicate.

(8) The effect of adding the cytokinin BAP (1mg/l), on root induction during a pulse exposure.

Shoot tips were collected from genotypes S8 and JK49. Shoots were placed in Petri dishes on QRC media containing 10mg/l IBA with or without 1mg/l BAP. The dishes were wrapped in aluminium foil to exclude any light, after six days in darkness the shoots were transferred to QRC media with charcoal. Each treatment had 12 replicates per genotype with two individuals per replicate.

(9) The effect of the culture medium from the micropropagation cycle (M91 and QRC with charcoal) on rooting capacity of shoots.

Shoot tips were collected from genotype S11 cultured on M91 medium and cultured on QRC medium with 3g/l activated charcoal. Shoots from each media were placed on QRC medium supplemented with 10mg/l IBA in Petri dishes. The dishes were wrapped in aluminium foil to exclude any light, after six days in darkness the shoots were transferred to QRC media with charcoal. Each treatment had 12 replicates with two individuals per replicate.

(10) The effect of different nutrient media formulations during a pulse exposure to IBA and darkness, on root induction.

Shoot tips were collected from genotype S11 and F5. Shoots from each genotype were placed in Petri dishes on media containing QRC salts, modified MS media salts, half strength MS media salts or modified WPM media salts supplemented with 10mg/l IBA. The dishes were wrapped in aluminium foil to exclude any light, after six days in darkness the shoots were transferred to QRC media with charcoal. Each treatment had 12 replicates per genotype with two individuals per replicate.

(11) The effect of taking shoots at different times after transfer on root induction.

Shoot tips from genotype S11 and selected genotype 49 were collected at increasing time intervals from the last subculture on QRC medium supplemented with 3g/l activated charcoal, starting at 4 weeks after the previous transfer. Shoots from each genotype were placed in Petri dishes containing QRC medium supplemented with 10mg/l IBA and 1mg/l BAP. The dishes were wrapped in aluminium foil to exclude any light, after six days in darkness the shoots were transferred to QRC media supplemented with 3g/l activated charcoal. Each treatment had 12 replicates per genotype with two individuals per replicate. The treatments were as follows: 4 weeks, 5 weeks, 7 weeks and 9 weeks after transfer of the cultures.

G. Experimental treatments for weaned plants

All plants used in the following experiments were weaned to the glasshouse by the method described in section E (unless stated otherwise).

(12) *The effect of weaning rooted and non-rooted shoots to the greenhouse on plant survival.*

At the end of each experiment (section 5. 5. F. (1 – 10)) both the rooted and non-rooted shoots were transferred to the glasshouse for weaning. The number of successfully weaned plantlets of each genotype was recorded two months after transfer to the greenhouse.

(13) *The effect of a cold period over winter after successful, weaning on bud break and shoot growth.*

The effect of giving the micropropagated plants a cold period after successful weaning to the glasshouse was tested. In December 2003, plants of genotype S8, S11, 47 and 49 weaned to the glasshouse in July 2003 were divided into two groups. The control group received the standard treatment of no cold period over winter by keeping them in a heated section of the glasshouse under lights while the cold treatment group were moved to a section of the glasshouse receiving no artificial heat or light for three weeks then transferred to the natural conditions outside at Teagasc, Kinsealy. The number of plants from each genotype in both groups is outlined in table 5.1. Within each group all plants of the same genotype were contained in one seed tray. On the same date in May 2004 the plants were assessed for bud break using the scale outlined in figure 5.1 and the height of new shoot growth (mm) was measured for any shoots at level 5 bud break.

Table 5. 1. The number of individual plants of genotype S8, S11, 47 and 49 in each treatment used to test the effect of *a* cold period after successful weaning on bud break and shoot growth.

Genotype/ Treatment	*Control (no cold period)*	*Cold period*
S8	20	14
S11	19	28
47	18	28
49	14	9

(14) *The growth over two years, of micropropagated plants of three genotypes after successful weaning to the glasshouse and transfer to large Rootrainer® pots.*

Rooted and non-rooted plantlets of genotype S11, F5 and 47 were transferred to the greenhouse in January 2003 for weaning. Four months later in April 2003, after successful weaning, healthy rooted plants from each genotype were transferred individually into large Rootrainer® pots containing peat. The pots were arranged randomly in 4 crates, each crate representing one block in a randomised block design experiment. Each block contained 13-16 plants of genotype S11, 3-4 plants of genotype F5 and 7-9 plants of genotype 47. The plants were grown in a glasshouse without artificial light or heat and received overhead irrigation. Fertiliser (Osmocote® (12-14 month release) Scotts, Ohio, U. S. A.) was applied by top dressing plants at the start of the growing season in April 2004. The height (mm) of each plant was measured at regular intervals from May to October 2003 and from May to September 2004.

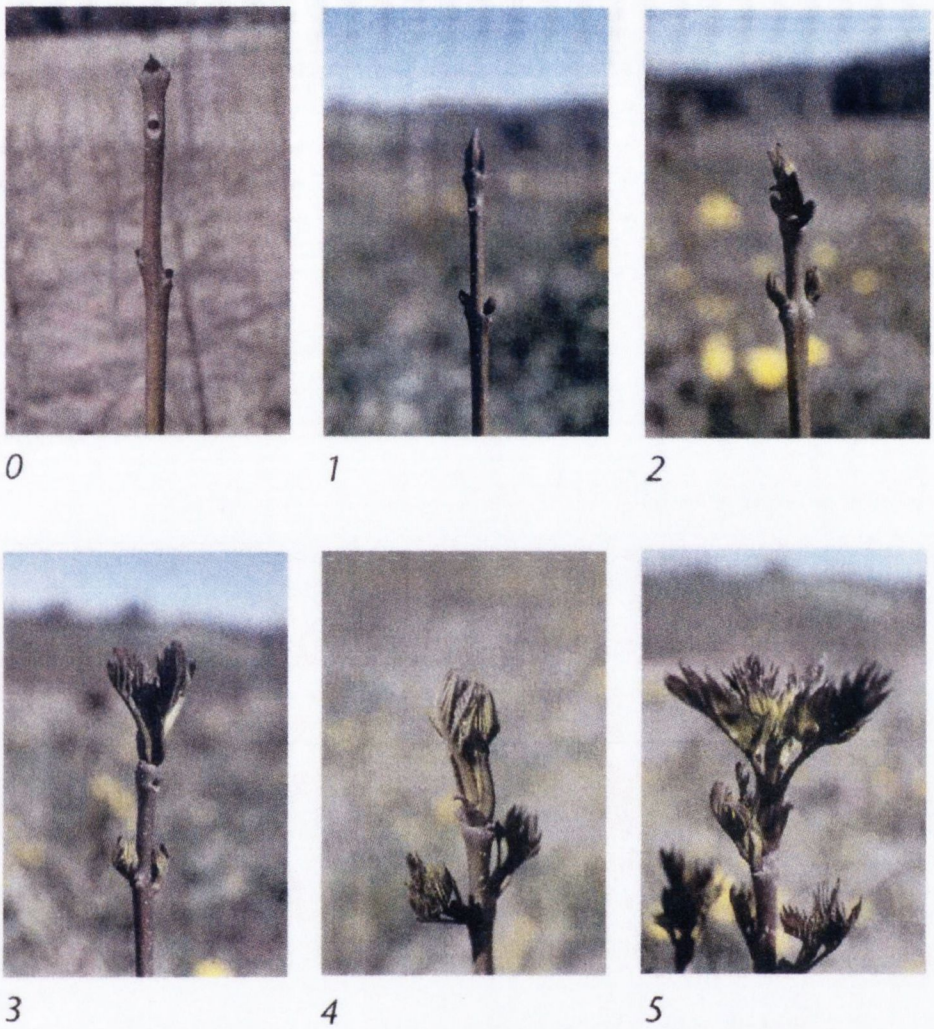
(15) *Root induction experiments on dormant cuttings from previously micropropagated plants and from grafted plants.*

In April 2004 cuttings were taken from micropropagated plants growing in the greenhouse and from grafted plants growing in field conditions at Teagasc, Kinsealy. All plants were nearing the end of dormancy and most buds were between level 0 and 1 of the bud opening categories described in figure 5. 1. Cuttings were approximately 10cm long and consisted of those with the apical bud and two sets of nodes (apical cuttings) and those with the apical bud removed and two sets of nodes (nodal cuttings). Apical and nodal cuttings were collected from micropropagated plants of genotype F5, 47 and 72, apical cuttings only were collected from shorter plants of genotype 49. To compare the responses of micropropagation derived cuttings with field derived cuttings, apical and nodal cuttings were collected from field grown grafted plants of genotype 47 and 49, apical cuttings only were collected from genotype 72. The appropriate treatment outlined in table 5. 2. was administered for each cutting type. All treatments contained 20 cuttings divided into four replicates of 5 cuttings randomised in Hassey trays filled with a mixture of medium grade peat: perlite (2:1 v/v). Trays were placed on a heated bench (20±2°C) under a plastic film in the nursery. After six weeks, recordings were made of the number of rooted cuttings, root number and the presence or absence of callus on the base of the cutting.

Table 5. 2. The root induction treatments administered to stem cuttings of dormant micropropagated and grafted plants.

	Treatment	*Cutting Type**
1	Control	A and N
2	Seradix® 3** powder (contains 8000ppm IBA) applied to base	A and N
3	8000ppm IBA in lanolin applied to the apical bud	A only
4	8000ppm IBA in lanolin applied to the apical cut surface	N only
5	8000ppm IBA in lanolin applied to the cutting base	A and N

* A, apical cutting and N, nodal cutting **Seradix® 3 (Hortichem, Wiltshire, U. K.)



F

Figure 5. 1. The bud break scale used for assessing the stage of bud opening in micropropagated plants weaned to the greenhouse.

5. 6. Results

(1) The effect of continuous exposure to different IBA concentrations.

The effect on rooting of continuous exposure to IBA at different concentrations, in the rooting media, was studied using shoot tips and nodes from genotypes S11 and 49 (section 5. 5. F. (1)). There was no significant difference in rooting between shoot tips (17.68%) and nodes (11.48%) (Chi square test, p=0.1346). Nodes showed a similar rooting response to shoot tips at the IBA concentrations tested. So, for further analysis the results from the shoot tips and nodes were grouped together and analysed as one data set. Shoots of genotype S11 had significantly higher rooting percentages *in vitro* compared to those of genotype 49 (25% and 5.13%, respectively) (data not shown on table) (Chi square test, p<0.0001).

The highest rooting percentage was obtained without IBA in the media (table 5. 3) for each genotype tested. Furthermore, adding IBA to the medium reduced rooting in both genotypes. The percentage of rooted shoots decreased as IBA level increased (table 5. 3). The combined rooting percentage of both genotypes declined significantly from 38.5% (0mg/l IBA) to 4.8% (20mg/l IBA) as IBA concentration increased (Tukey's HSD, p< 0.0001) (table 5. 3). Genotype S11 had a higher rooting capacity compared to genotype 49 at each IBA level tested (causing a significant interaction) (Factorial ANOVA, p<0.006) (figure 5. 2, table 5. 3).

It was noted that the percentage of shoots with callus > 5mm in diameter significantly increased from the control treatment of 0 mg/l IBA (0% callus) to the treatments exposed to IBA at various concentrations (total values range from 59.7% - 75.6% callus) (Tukey's HSD, p< 0.0001) (table 5. 4). There was a significant difference in the occurrence of callus on shoots of different genotype, genotype S11 had the highest percentage of shoots with callus (69.5%) compared to 49 (35.9%) (data not shown on table) (Chi square test, p<0.0001). A significant interaction between IBA concentration, genotype and the presence of callus was identified (Factorial ANOVA, p<0.00001) (table 5. 4). The occurrence of callus > 5mm diameter was significantly less on rooted shoots (32.65%) than on non-rooted shoots (56.8%) (Chi square test, p< 0.02).

Table 5. 3. Percentage rooting in genotypes S11 and 49 at different concentrations of IBA (mg/l).

IBA (mg/l)	*Genotype*		*Total***
	S11	*49*	
0	60%^{aA*}	15.9%^{aB}	38.5%^{a}
5	22.2%^{bA}	5%^{aA}	13.2%^{b}
10	13.6%^{bA}	0%^{aA}	7.3%^{b}
15	20%^{bA}	2.5%^{aA}	8.3%^{b}
20	7.1%^{bA}	0%^{aA}	4.8%^{b}

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference (p<0.003) according to Tukey's HSD. ** Data from both genotypes combined.

Table 5. 4. Percentage callus > 5mm diameter in genotypes S11 and 49 at different concentrations of IBA (mg/l).

IBA (mg/l)	*Genotype*		*Total***
	S11	*49*	
0	0%^{aA*}	0%^{aA}	0%^{a}
5	100%^{bA}	50%^{bB}	73.7%^{b}
10	97.7%^{bA}	50%^{bB}	75.6%^{b}
15	95%^{bA}	60%^{bB}	71.7%^{b}
20	80.9%^{bA}	15%^{aB}	59.7%^{b}

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.04$) according to Tukey's HSD. ** Data from both genotypes combined.

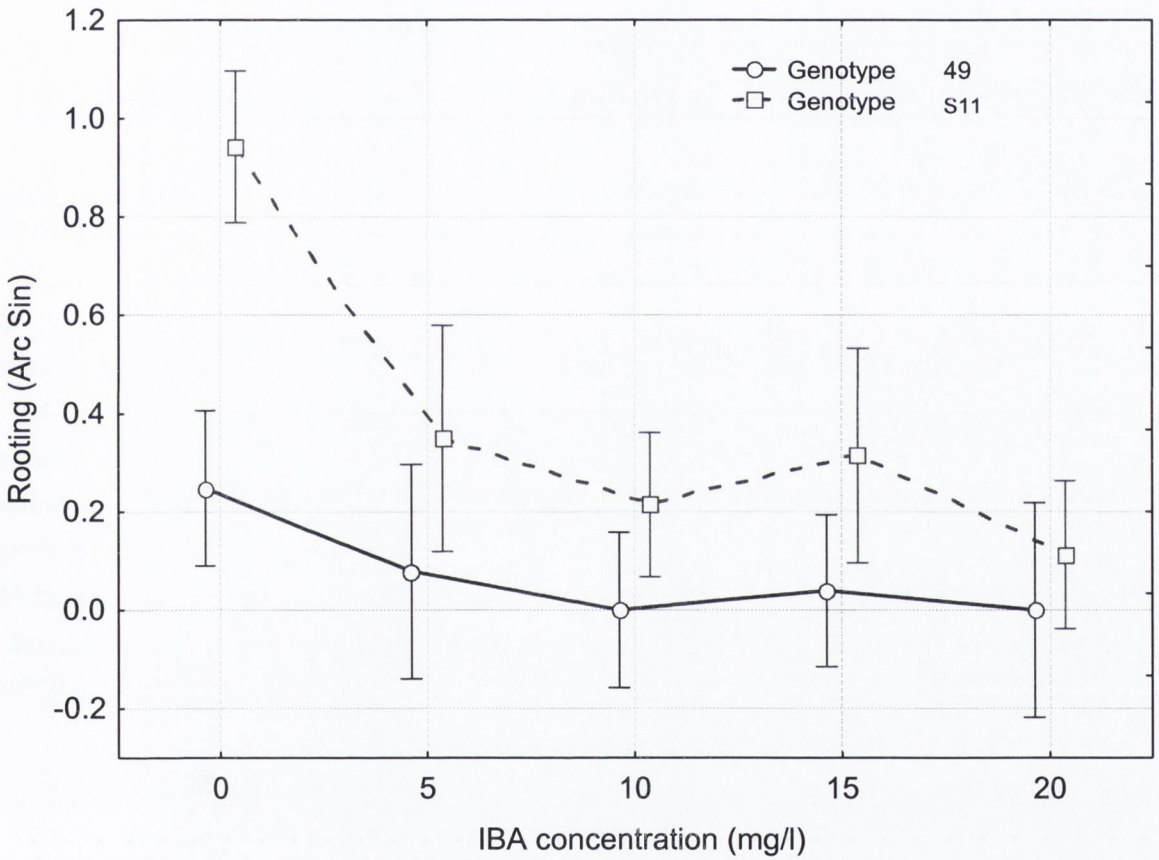


Figure 5. 2. The interaction between IBA concentration, genotype and rooting (vertical bars denote 95% confidence interval).

(2) The effect of a dark period of 0, 2, 4, 8 and 10 days during continuous exposure to IBA on root induction.

Shoot tips of genotype S11 and 49 were used to test the effect of placing shoots in the dark for different time periods during continuous exposure to IBA, on root induction (section 5. 5. F (2)). Shoots of genotype 49 failed to produce any roots in any treatment during this experiment. For genotype S11 there was no significant difference in rooting over the different dark periods, however rooting increased from 25% at 0 days to 45% at 10 days in the dark (table 5. 5) (ANOVA, p=0.167).

Table 5. 5. Effect of dark periods (days) on percentage rooting for genotype S11.

Days in darkness	*Rooting (%)*
0	25*
2	15
4	20
8	40
10	45

*No significant difference between days in darkness (ANOVA, p=0.167).

(3) The effect of a dark period and pulse exposure for 0, 2, 4 and 8 days to IBA.

Shoot tips were of genotype F5 were used to test the effects of a dark period combined with pulse exposure to IBA for 0 to 8 days (section 5. 5. F. (3)). Genotype F5 had an overall rooting percentage of 20.7% in this experiment. Rooting was significantly higher in the dark administered treatments (31.7%) than in the light treatments (11.7%) (Chi square test, $p<0.008$) (table 5. 6). The number of days spent on IBA had no significant effect on rooting (Chi square test, p=0.3469), however rooting increased from 15% at 0 days on IBA to 30% at 8 days on IBA medium. In the dark treatments, rooting increased from 15% at day 0 to 40% at 8 days (table 5. 6). There was no significant interaction between light or dark treatment, and days on IBA and rooting (day 0 treatment excluded from analysis) (Factorial ANOVA, p=0.5417).

Table 5. 6. Effects of pulse exposure to IBA (1mg/l) in darkness for 0 – 8 days on rooting of genotype F5.

Days	*Treatment*		*Total****
	Light	*Dark*	
0	15%[a*]		15%[a*]
2	10%[aA]	20%[aA]	15%[a]
4	5%[aA]	35%[aA]	20%[a]
8	20%[aA]	40%[aA]	30%[a]
Total****	11.7%[A**]	31.7%[B]	20.7%

* No significant differences at the 5% level according to Tukey's HSD. **Total percentage rooting in light (A) and dark treatments (B) significantly different (Chi square test, $p<0.008$). *** Data from both genotypes combined. ****Data from all treatments combined.

Statistical inferences could not be made for root number or length within treatments due to the small number of individuals involved however, total root length was significantly higher in the light treatments (35.8 mm) compared to the dark (16.8 mm) (ANOVA, $p<0.01$).

(4) The effect of pulse treatment of two days versus continuous exposure to IBA at different concentrations.

Shoot tips from genotype S8 and 47 were used to test the effect of a pulse treatment of two days to IBA compared to a continuous treatment with IBA, on root induction (section 5. 5. F. (4)). Shoots of each genotype cultured continuously in the presence of IBA (10 and 20mg/l) failed to produce any roots (data not shown) but did produce callus (data shown in table 5. 8). The data for shoots exposed to IBA for a pulse of two days are summarised in table 5. 7. Significantly more shoots produced roots in the pulse treatment (21.7%) compared to the continuous exposure treatment (0%, data not shown) (Chi square test, $p<0.0001$). Highest rooting was obtained using a pulse treatment (two days) with IBA at 20mg/l. However, IBA concentration had no significant effect on the percentage of shoots rooting in the pulse treatment (One-way ANOVA S8: $p=0.36$, 47: $p=0.27$, combined: $p=0.16$) however, rooting increased with IBA level in both genotypes (see table 4.1). There was a significant difference in percentage rooting between genotypes S8 (2.8%) and 47 (21.3%) (Chi square test, $p<0.0001$). In the pulse treatment there was no significant interaction between IBA level, genotype and rooting (Factorial ANOVA, $p=0.67$).

Table 5. 7. The effects of pulse treatment with IBA for two days on percentage rooting for genotypes S8 and 47.

IBA (mg/l)	*Genotype*		*Total***
	S8	*47*	
0	0%^{aA*}	25%^{aA}	12.5%^{a}
10	5%^{aA}	40%^{aB}	22.5%^{a}
20	10%^{aA}	50%^{aB}	30%^{a}
Total***	2.8%^{A}	21.3%^{B}	21.7%

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.05$) according to Tukey's HSD. ** Data from both genotypes combined. ***Data from all treatments combined.

Callus production was compared for shoots exposed continuously to IBA with those given a two day pulse treatment (data not shown). Overall, continuous exposure resulted in 46.8% of shoots with callus > 5mm diameter whereas the total over all pulse treatments was 5% (Chi square test, $p< 0.0001$). The data for the pulse treatments are not shown. A summary of the effects of IBA on callus production in the continuous exposure treatments is shown in table 5. 8. Rooted shoots did not produce callus whereas 26.6% of non-rooted shoots produced callus (Chi square test, $p=0.003$) (data not shown). In the continuous treatment, the number of shoots with callus was significantly higher in shoots exposed to IBA (10mg/l: 73.3%, 15mg/l: 68.75%) compared to the control (0mg/l IBA: 0%) (Tukey's HSD, $p=0.0001$) (table 4. 2). There was no significant difference between genotype and the occurrence of callus (Chi square test, $p=0.80$). There was no significant interaction between IBA concentration, genotype and the occurrence of callus (Factorial ANOVA, $p=0.27$) (table 5. 8).

Table 5. 8. Effect of continuous exposure to IBA on callus (> 5mm diameter) production in genotypes S8 and 47.

IBA (mg/l)	*Genotype*		*Total***
	S8	*47*	
0	0%^{aA*}	0%^{aA}	0%^{a}
10	57%^{bA}	87.5%^{bA}	73.3%^{b}
15	62.5%^{bA}	75%^{bA}	68.7%^{b}

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.002$) according to Tukey's HSD. ** Data from both genotypes combined.

(5) The effect of a pulse exposure for two days in darkness at different IBA concentrations on root induction in two ash genotypes.

Shoot tips from genotype S11 and 47 were used to further test the effect of different concentrations of IBA during a pulse treatment (section 5. 5. F. (5)). A significantly higher percentage of shoots rooted from genotype S11 (83.4%) compared to genotype 47 (67.2%) (Chi square test, $p=0.02$). There was no significant

difference in rooting at different IBA concentrations for both genotypes combined (Chi square test, p=0.76). However, in genotype 47 rooting increased from 50% at 0 mg/l IBA to 85% at 20 mg/l IBA (table 5. 9).

Table 5. 9. The effects of pulse exposure to IBA for two days in darkness on percentage rooting in genotypes S11 and 47.

IBA (mg/l)	*Genotype*		*Total***
	S11	*47*	
0	93% *	50%*	71.9%*
5	87.5%	68.7%	78.1%
10	68.7%	75%	71.9%
20	85.7%	85%	81.2%

* No significant differences at the 5% level according to Tukey's HSD. ** Data from both genotypes combined.

Rooted shoots of genotype S11 produced significantly more roots than genotype 47 (4.6 roots/shoot and 2.1 roots/shoot respectively) (ANOVA, $p<0.00001$) and there was a significant difference between IBA concentration and the number of roots produced by the shoots (ANOVA, $p=0.04$) (table 5. 10 (a) and (b)). A significant interaction exists between genotype, IBA concentration and root number (Factorial ANOVA, $p=0.01$) (table 5. 10 (a) and (b)). The total length of roots from genotype S11 were significantly longer than that of 47 (47.1mm and 21.9mm respectively) (ANOVA, $p=0.0003$). There was a significant interaction between genotype, IBA concentration and total root length (Factorial ANOVA, $p<0.01$). There was no significant difference in mean root length between both genotypes (S11: 10.1mm and 47: 11.6mm) (ANOVA, $p=0.26$).

Table 5. 10. (a) The number of roots per shoot, total root length and mean root length in genotype S11 after pulse exposure to IBA at different concentrations (mg/l).

IBA (mg/l)	No. roots/ shoot ± S. E. *	Total root length ± S. E. (mm)	Mean root length ± S. E. (mm)
0	3.0 ± 0.4^{a***A***}	26.9 ± 3.7^{aA}	9.1 ± 0.5^{aA}
5	4.1 ± 0.6^{abA}	40.6 ± 7.7^{aA}	10.2 ± 1.5^{aA}
10	7.1 ± 0.6^{bA}	86.5 ± 20.0^{bB}	12.4 ± 2.6^{aA}
20	5.0 ± 0.7^{abA}	44.4 ± 7.6^{aA}	9.3 ± 1.3^{aA}

* ± Standard error. ** Different letters within columns (lowercase) indicate a significant difference ($p<0.05$) according to Tukey's HSD. ***The uppercase letters refer to differences between the measured variables between table 5. 10 (a) and (b) e.g. no. of roots/ shoot for genotype S11 and genotype 47 for 0mg/l IBA carry the same letter A, therefore these are not statistically different according to Tukey's unequal N HSD.

Table 5. 10. (b) The number of roots per shoot, total root length and mean root length in genotype 47 after pulse exposure to IBA at different concentrations (mg/l).

IBA (mg/l)	No. roots/ shoot ± S. E. *	Total root length ± S. E. (mm)	Mean root length ± S. E. (mm)
0	1.1 ± 0.1^{a**A***}	21.6 ± 8.1^{aA}	21.2 ± 8.2^{aA}
5	1.9 ± 0.2^{abA}	18.5 ± 3.5^{aA}	9.8 ± 1.5^{aA}
10	2.1 ± 0.3^{bB}	22.6 ± 4.1^{aA}	10.6 ± 1.3^{aA}
20	3.2 ± 0.5^{bA}	24.6 ± 4.7^{aA}	7.7 ± 1.1^{aB}

* ± Standard error. ** Different letters between genotypes within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.05$) according to Tukey's HSD. ***The uppercase letters refer to differences between the measured variables between table 10. 2 (a) and (b) e.g. no. of roots/ shoot for genotype S11 and genotype 47 for 0mg/l IBA carry the same letter A, therefore these are not statistically different according to Tukey's unequal N HSD.

(6) The effect of a pulse treatment for two days on 10mg/l IBA on two genotypes.

Shoot tips of genotype F5 and 70 were used to test the effect of a pulse treatment on IBA medium on root induction (section 5. 5. F. (6)). There was no significant difference in rooting percentages for F5 (53.13%) and genotypes 70 (42.8%) (Chi square test, $p=0.43$). Mean root length was significantly higher in F5 (4.7mm) compared to genotype 70 (6.8mm) (ANOVA, $p=0.006$). Total root length and the number of roots per shoot were not significantly different (ANOVA, $p=0.50$ and $p=0.39$, respectively) (table 5. 11).

Table 5. 11. The number of roots per shoot, total root length and mean root length in genotypes 70 and F5 after pulse exposure to 10 mg/l IBA for two days.

Genotype					
70			*F5*		
No. roots/ shoot ± S. E. *	Total root length ± S. E. (mm)	Mean root length ± S. E. (mm)	No. roots/ shoot ± S. E.	Total root length ± S. E. (mm)	Mean root length ± S. E. (mm)
1.7 ± 0.25^{a**}	10.5 ± 0.8^{a}	6.8 ± 0.7^{a}	1.5 ± 0.12^{a}	6.9 ± 0.5^{b}	4.7 ± 0.3^{b}

* ± Standard error. ** Different letters between genotypes in each category indicate a significant difference ($p<0.05$) according to one way ANOVA.

(7) The effect of three media darkening methods on rooting using a continuous exposure to IBA.

Shoot tips from genotypes S8 and 49 were used to test the effect of media darkening on root induction (section 5. 5. F. (7)). There was no significant difference in percentage rooting between the genotypes S8 (30%) and 49 (38%) (Chi square test, $p=0.26$) (table 5. 12). In genotype S8 the percentage of shoots rooting was increased by darkening the media. However, there was no significant difference in rooting between all four treatments (One-way ANOVA, $p=0.24$) (table 5. 12). In genotype 49 there was a significant increase in percentage rooting when charcoal was added to the media compared to the other treatments (One-way ANOVA, $p<0.0001$) (table 5. 12). There was a significant interaction between genotype, media darkening method and rooting (Factorial ANOVA, $p<0.0001$), both genotypes had similar percentage rooting in the

control and foil darkened treatments but genotype S8 had the highest rooting in the dye darkened media (45%) and genotype 49 had highest rooting in the charcoal darkened treatment (87%) (table 5.12, figure 5. 3).

Table 5. 12. Percentage rooting in genotypes S8 and 49 after three media darkening methods during continuous exposure to IBA.

Media darkening	*Genotype*		
	S8	*49*	*Total***
Control	17%^{aA*}	41%^{abA}	27%^{a}
Foil	29%^{aA}	32%^{aA}	31%^{ab}
Dye	45%^{aA}	8%^{aA}	25%^{a}
Charcoal	31%^{aA}	87%^{bB}	59%^{b}
Total***	30%^{A}	38%^{A}	

* Different letters within columns (lowercase) and rows (uppercase) indicate a significant difference ($p<0.05$) according to Tukey's HSD. ** Data from both genotypes combined. ***Data from all treatments combined.

In both genotype S8 and 49 there was no significant differences in the number of roots produced by the rooted explants in each treatment (One-way ANOVA, S8 $p=0.14$, 49 $p=0.05$) (table 5. 13). Total root length and mean root length were significantly higher in the charcoal treatment compared to the other treatments in both genotypes (total root length: One-way ANOVA, S8 $p<0.0004$, 49 $p<0.0003$; mean root length: One-way ANOVA, S8 $p<0.00006$, 49 $p<0.00004$) (table 5. 13). The dye treatment category in genotype 49 was excluded from statistical analysis for number of roots, total root length and mean root length due to the small number of rooted plants. It was noted at the time of recording that a high number of shoots of genotype 49 in the dye treatment had developed callus growth at the base of their stems.

Table 5. 13. (a) The number of roots per shoot, total root length and mean root length in genotype S8 three media darkening methods during continuous exposure to IBA.

Media darkening	No. roots/ shoot ± S. E.*	Total root length ± S. E. (mm)	Mean root length ± S. E. (mm)
Control	2.8 ± 0.9 ^{***A***}	12.3 ± 2.7^{aA}	5.5 ± 1.4^{aA}
Foil	2.7 ± 0.6 ^{aA}	14.8 ± 2.9^{aA}	6.5 ± 1.6^{aA}
Dye	1.6± 0.3 ^{aA}	5.2 ± 1.3^{a}	3.2 ± 0.4^{a}
Charcoal	1.2± 0.2 ^{aA}	49.2 ± 15.8^{bA}	40.5 ± 12.7^{bA}

* ± Standard error. ** Different letters within columns (lowercase) indicate a significant difference ($p<0.05$) according to Tukey's unequal N HSD. ***The uppercase letters refer to differences between the measured variables between table 5. 13 (a) and (b), different letters indicate a significant difference according to Tukey's unequal N HSD.

Table 5. 13. (b) The number of roots per shoot, total root length and mean root length in genotype 49 three media darkening methods during continuous exposure to IBA.

Media darkening	No. roots/ shoot ± S. E.*	Total root length ± S. E. (mm)	Mean root length ± S. E. (mm)
Control	2.1 ± 0.5 ***A***	11.3 ± 2.3^{aA}	5.9 ± 1.4^{aA}
Foil	3.0 ± 0.3 ^{aA}	27.6 ± 7.8^{aA}	9.1 ± 2.6^{aA}
Dye	1.0 ± 0.0 ^{aA}	5.5 ± 3.5	5.5 ± 3.5
Charcoal	2.1 ± 0.3 ^{aA}	90.1 ± 14.5^{bA}	48.4 ± 7.4^{bA}

* ± Standard error. ** Different letters within columns (lowercase) indicate a significant difference ($p<0.05$) according to Tukey's unequal N HSD. ***The uppercase letters refer to differences between the measured variables between table 5. 13 (a) and (b), different letters indicate a significant difference according to Tukey's unequal N HSD. The dye treatment in genotype 49 was excluded from any analysis.

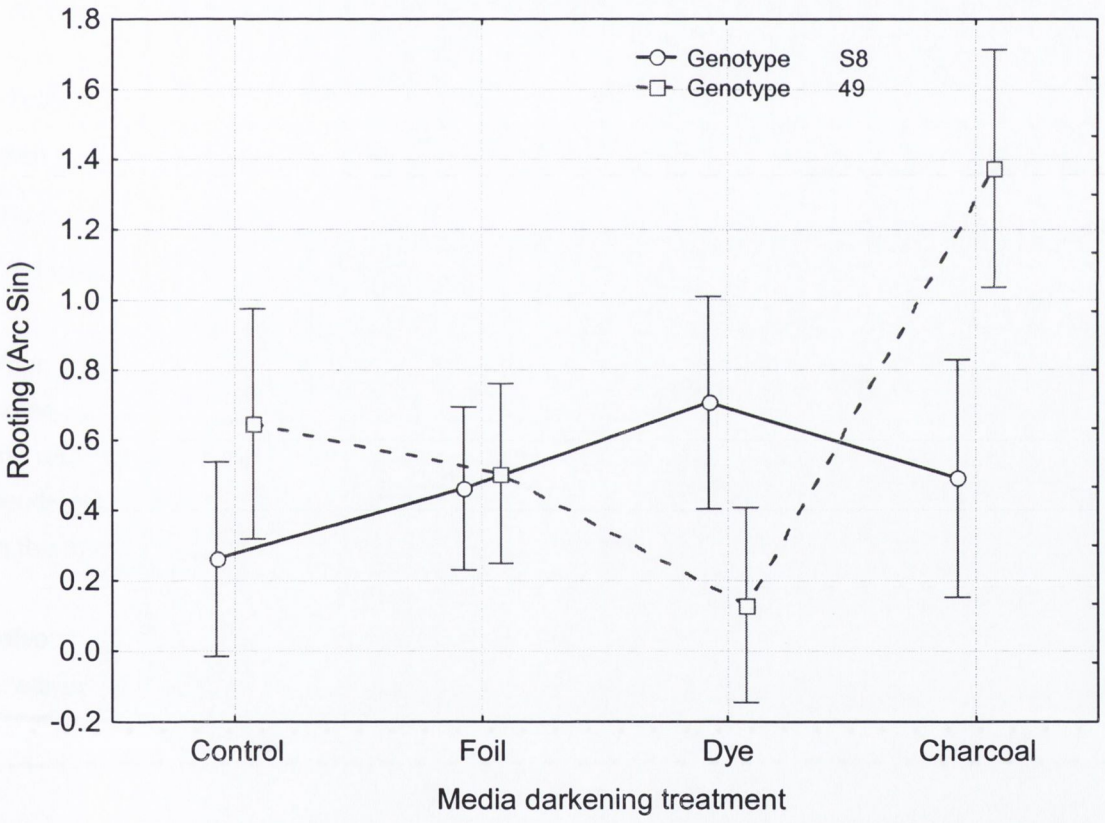


Figure 5. 3. The interaction between genotype, media darkening treatment and rooting (vertical bars denote 95% confidence interval).

(8) The effect of adding the cytokinin BAP (1mg/l), on root induction during a pulse exposure.

Shoot tips from genotype S8 and 49 were used to test the effect of adding the cytokinin BAP to the root induction media (section 5. 5. F. (8)). Significantly more shoots rooted from genotype 49 (58.1%) compared to genotype S8 (33.3%) (Chi-square test, $p<0.016$). Shoots from both genotypes were more likely to produce roots on the media with BAP compared to the media without BAP (table 5. 14). Due to excess fungal contamination in the media without BAP, the majority of shoots in this treatment had to be discarded and only small numbers remained in this treatment at the end of the experiment. No statistical tests were done on the individual genotype data displayed in Table 5. 14.

Table 5. 14. Rooting data for shoots of genotype S8 and 49 placed on root induction media with or without 1mg/l BAP.

	Genotype			
	S8		*49*	
	With BAP	Without BAP	With BAP	Without BAP
Rooting (%)	35.1*	28.6	62.2	33.3
No. roots**	1.5 ± 0.4	2.7 ± 0.2	2.0 ± 0.3	1.5 ± 0.5
Total root length (mm) **	21.4 ± 3.1	20.8 ± 6.6	20.1 ± 2.2	25.7 ± 9.2
Mean root length (mm)**	29.6 ± 7.4	60.0 ± 27.6	23.8 ± 5.0	34.0 ± 1.0

* No statistical tests done on all variables due to low N numbers in the media without BAP.

** ± Standard error.

When the data from both genotypes are combined there is no significant difference in the percentage of rooted shoots on either media with or without BAP (48.6 and 30.0%, respectively) (Chi-square test, $p=0.136$). The remaining variables were not statistically analysed due to low N numbers in the media without BAP, the results are reported in table 5. 15. The number of roots, total root length and mean root length were higher on the media without BAP (table 5. 15).

Table 5. 15. Rooting data for shoots of genotype S8 and 49 (combined) placed on root induction media with or without 1mg/l BAP.

	With BAP	*Without BAP*
Rooting (%)	48.6*	30.0*
No. roots ± S. E.**	1.8 ± 0.2***	2.3± 0.8
Total root length ± S. E. (mm)	20.5 ± 1.8	22.5 ± 4.9
Mean root length ± S. E. (mm)	25.3 ± 4.2	51.3 ± 18.3

*There were no significant differences at the 5% level according to the Chi-square test. **± Standard error. ***No statistical tests done on the variables No. roots, total root length of mean root length due to low N numbers in the without BAP media.

The experiment was repeated using only shoots from genotype 49. The results are shown in table 5. 15. The percentage of shoots rooting was slightly higher on media with BAP however, there was no significant difference in the percentage of shoots rooting, number of roots, total root length or mean root length (Chi-square test, p=0.88; One-way ANOVA, p=0.34, p=0.70 and p=0.09).

Table 5. 16. Rooting data for shoots of genotype 49 placed on root induction media with or without 1mg/l BAP.

	With BAP	*Without BAP*
Rooting (%)	58.3*	55.6
No. roots ± S. E. **	2.0 ± 0.3***	1.6 ± 0.3
Total root length (mm) ± S. E.	18.6 ± 6.6***	22.4 ± 6.7
Mean root length (mm) ± S. E.	15.9 ± 2.9 ***	25.5 ± 3.9

*There were no significant differences at the 5% level according to the Chi-square test. **± Standard error. *** There were no significant differences at the 5% level according to One-way ANOVA.

(9) The effect of the culture medium of the micropropagation cycle (M91 and QRC with charcoal) on rooting capacity of shoots.

Shoot tips from genotype S11 were used to test the effect of taking shoots from modified MS medium compared to taking shoots from the QRC medium with activated charcoal on root induction (section 5. 5. F. (9)). The results are shown in table 5. 17. There was no significant difference between the percentage of shoots rooting from either the modified MS media or the QRC media with activated charcoal (Chi-square test, p=0.13). The number of roots per rooted shoot, total root length and mean root length were similar for shoots taken from both media tested (One-way ANOVA, p=0.99, p=0.35 and p=0.33, respectively).

Table 5. 17. The effect of taking shoots for rooting from M91 medium (contains PGRs) compared to those taken from QRC medium with activated charcoal (no PGRs).

	M91 medium (with PGRs)	*QRC media with charcoal (without PGRs)*
Rooting (%)	80*	68.6*
No. roots ± S. E. **	2.5 ± 0.2***	2.5 ± 0.2
Total root length (mm) ± S. E.	72.9 ±9.1***	85.2 ±8.5
Mean root length (mm) ± S. E.	33.2 ± 2.5***	36.8 ± 2.6

*There were no significant differences at the 5% level according to the Chi-square test. **± Standard error. *** There were no significant differences at the 5% level according to One-way ANOVA.

(10) The effect of nutrient media formulations during a pulse exposure to IBA and darkness, on root induction.

Shoot tips from genotype S11 and F5 were used to test the effect of four different nutrient media formulations in the rooting medium on root induction (section 5. 5. F. (10)). The results are shown in table 5.

18. Genotype S11 had a significantly higher number of shoots producing roots compared to genotype F5 (68.6 and 46.6%, respectively) (Chi-square test, $p<0.008$). The QRC media salt treatment for genotype S11 was excluded from all analysis due to contamination. There were no significant differences in rooting percentage between the media tested (Chi square test, S11 $p=0.19$, F5 $p=0.34$ and total $p=0.06$). The highest rooting percentages for both genotypes, was recorded on the rooting media containing modified WPM salts. There was no significant interaction for rooting between both genotypes and the four media tested (Factorial ANOVA, $p=0.31$).

Table 5. 18. The effect of four different nutrient media formulations in the rooting medium, on percentage rooting.

Nutrient media	*Genotype*		
	S11	*F5*	*Total***
QRC	33.3%*	53.3%*	47.6%*
Modified MS	60.0%	36.3%	47.6%
Half strength modified MS	72.0%	41.6%	57.1%
WPM	87.5%	66.6%	78.6%

* There were no significant differences at the 5% level according to the Chi-square test. ** Data combined from both genotypes

There was no significant difference in the number of roots produced by shoots of either genotype S11 or F5 on the four nutrient media tested (One-way ANOVA, S11 $p=0.86$, F5 $p=0.06$ and total $p=0.05$, table 5. 19). There was no significant interaction between genotype and media for root number (Factorial ANOVA, $p=0.92$).

Table 5. 19. The effect of four different nutrient media formulations in the rooting media, on root number.

Nutrient media	*Genotype*		
	S11	*F5*	*Total**
QRC	1.5**± 0.5***	1.2 ± 0.2	1.3 ± 0.1
Modified MS	2.4 ± 0.3	1.5 ± 0.2	2.0 ± 0.2
Half strength modified MS	2.6 ± 0.4	1.8 ± 0.3	2.3 ± 0.3
WPM	2.6 ± 0.2	2.1 ± 0.2	2.4 ± 0.2

* Data combined from both genotypes. ** There were no significant differences at the 5% level according to ANOVA. *** ± Standard error.

The data for total root length was square root transformed, prior to analysis. There was no significant difference in the length of roots produced on any of the four media tested for both genotypes (One-way ANOVA, S11 $p=0.42$ and F5 $p=0.06$). When the data was combined, a significant difference was identified between media salt and total root length (One-way ANOVA, $p<0.03$). However, the differences between means could only be separated by Fisher's post-hoc test and were not recognised by Tukey's test (Table 5.

20). There was no significant interaction between genotype and nutrient media for total root length (Factorial ANOVA, p=0.96).

Table 5. 20. The effect of four different nutrient media formulations in the rooting media, on total root length (mm).

Nutrient media	*Genotype*		
	S11	*F5*	*Total*
QRC	56.0*± 31.0**	26.1 ± 7.0*	32.1 ± 8.1a***
Modified MS	71.2 ± 19.0	20.6 ± 4.0	50.9 ± 21.7ab
Half strength modified MS	93.0 ± 13.8	39.3 ± 7.1	73.8 ± 10.3b
WPM	91.6 ± 13.8	45.6 ± 11.0	74.9 ± 10.6b

* There were no significant differences at the 5% level according to ANOVA. ** ± Standard error. *** Different letters within columns indicate a significant difference according to Fisher's LSD p<0.02.

(11) The effect of taking shoots at different times after transfer on root induction.

Shoot tips from genotype S11 and 49 were collected at various time intervals to test the effect of time after transfer on root induction. The results for genotype S11 are shown in table 5. 21. There was no significant difference in the percentage of shoots rooting over the 5 week period (Chi square test, p=0.96) however, rooting did decrease by 6% from week 4 to week 9. There was no significant variation in any other variable recorded including root number and mean root length (mm), only total root length (mm) showed any variation over the five week period (One-way ANOVA, p=0. 24, p=0.08 and p<0.01, respectively).

Table 5. 21. The effect on root induction, of taking shoots from genotype S11 at 4, 5, 7 and 9 weeks after transfer to QRC medium with charcoal.

	Week			
	4	*5*	*7*	*9*
Rooting (%)	83.3a**	80.0a	79.2a	77.3a
No. Roots**	2.5 ± 0.3a	3.0 ± 0.3a	2.2 ± 0.3a	2.7 ± 0.3a
Total root length (mm) **	92.0 ± 17.2ab	79.1 ± 14.4ab	54.5 ± 11.9a	134.2 ± 20.3b
Mean root length (mm) **	41.2 ± 4.5a	35.2 ± 4.9a	30.4 ± 4.8a	49.8 ± 6.8a

* Different letters between columns indicate a significant difference according to Tukey's HSD at the 5% level. ** ± Standard error.

The results for genotype 49 are shown in table 5. 22. There was no significant difference in the percentage of shoots rooting over the 5 week period (Chi square test, p=0.62) however, rooting did increase by approximately 12% from week 4 to week 9. There was no significant variation in the number of roots produced by shoots between the weeks tested, the total root length or mean root length (One-way ANOVA, p=0.61, p=0.53 and p=0.05, respectively).

Table 5. 22. The effect on root induction, of taking shoots from genotype 49 at 4, 5, 7 and 9 weeks after transfer to QRC medium with charcoal.

	Week			
	4	*5*	*7*	*9*
Rooting (%)	29.2*	45.8	33.3	41.7
No. Roots**	2.6 ± 0.6	2.4 ± 0.4	1.7 ± 0.4	2.2 ± 0.3
Total root length (mm) **	14.5 ± 6.5	51.3 ± 10.9	69.0 ± 18.5	13.7 ± 4.2
Mean root length (mm) **	18.5 ± 3.7	20.2 ± 3.4	38.2 ± 6.4	14.5 ± 1.7

* No significant differences ($p \leq 0.05$) recorded for any variable measured. ** ± Standard error.

At the end of the rooting experiments, a final analysis was undertaken to demonstrate that in the experiments shoots taken from cultures initiated from seed (juvenile material) were more likely to root than those taken from cultures derived from mature trees. The rooting of approximately 500 shoots of each type of material (juvenile and mature) exposed to similar experimental conditions was analysed by Chi square test. The result of the analysis showed that a significantly higher proportion of juvenile material (23.1% rooting) produced roots in the experiments compared to that of the mature shoot material (15.5% rooting) (Chi Square test $p < 0.003$).

(12) *The effect on plant survival of weaning rooted and non-rooted shoots to the greenhouse.*

At the end of each experiment, both the rooted and non-rooted shoots were weaned to the glasshouse (section 5. 5. G. (12)). The results for the survival of plants from each genotype are shown in table 5. 23. For all genotypes, the rooted plantlets were more likely to survive the transfer to the glasshouse than the non-rooted plantlets (Two sided T-test, $p<0.01$, for all comparisons). For the rooted plants survival rates ranged significantly from 61.7% in genotype S8 to 95.6% in genotype 47 (Two sided T-test, $p<0.006$). For the non-rooted plants survival rates ranged significantly from 17.8% in genotype 49 to 62.5% in genotype F5 (Two sided T-test, $p<0.0001$).

Table 5. 23. The survival rate (%) of rooted and non-rooted shoots of genotype S8, S11, F5, 47 and 49 when weaned to the glasshouse.

Genotype	*Rooted (in vitro)*	*Non-rooted (in vitro)*
S8	61.7% (34*)[a]**	33.9% (162)[b]
S11	80.3% (178)[a]	20.3% (227)[b]
F5	91.2 % (34)[a]	62.5% (24)[b]
47	95.6% (23)[a]	32.9% (82)[b]
49	80.3 % (61)[a]	17.8% (292)[b]
Total	80.6% (330)[a]	24.7% (787)[b]

* The value in parentheses indicates the total number of shoots transferred to the glasshouse. ** Different letters between columns indicate a significant difference according to a two-sided Students T-test, $p<0.01$.

(13) *The effect of a cold period after successful weaning on bud break and shoot growth.*

The effect of giving the micropropagated plants a cold period after successful weaning to the glasshouse was tested on genotypes S8, S11, 47 and 49 (section 5. 5. G. (13)). The results are shown in table 5. 24. For all the genotypes, plants in the control group, that received no cold period, tended to be in the lower bud opening categories compared to plants in the group receiving cold period treatment. The mean bud opening category for the all genotypes in the control group (1.5) was significantly lower than that for the cold treatment group (3.7) (data not shown) (One-way ANOVA, $p<0.0001$). However, when the height of new growth from shoots in category 5 was collated for all genotypes and analysed (data not shown), there was no significant difference in shoot height between the control (5.7mm) and treatment groups (4.6mm) (One-way ANOVA, $p=0.29$).

Table 5. 24. The percentage of plantlets from each genotype in the bud opening categories (0 - 5) from the control and cold period treatments.

		Bud opening category					
Genotype	*Treatment*	*0*	*1*	*2*	*3*	*4*	*5*
S8	Control	73.7%	10.5%	0.0%	0.0%	0.0%	15.8%
	Cold period	0.0%	7.1%	0.0%	0.0%	28.6%	64.3%
S11	Control	35.0%	0.0%	0.0%	0.0%	0.0%	65.0%
	Cold period	7.1%	0.0%	0.0%	0.0%	0.0%	92.9%
47	Control	66.7%	0.0%	0.0%	11.1%	0.0%	22.2%
	Cold period	14.3%	17.9%	14.3%	10.7%	10.7%	32.1%
49	Control	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	Cold period	0.0%	22.2%	33.3%	44.4%	0.0%	0.0%

(14) *The growth of micropropagated plants of three genotypes after successful weaning to the glasshouse and transfer to large root trainer pots.*

Plantlets of genotype S11, F5 and 47 were used to assess the growth, in terms of plant height, of successfully weaned micropropagated plants over two consecutive years. The results are displayed graphically in figure 5. 4. At the final measurement in September 2004, 58 out of 59 (98.3%) plants of genotype S11, 24 out of 34 (70.6%) plants of genotype 47 and 11 out of 14 (78.6%) plants of genotype F5 were surviving. The plants in block 2 were significantly higher than those in block 4 (Tukey's unequal N HSD, $p<0.003$). At the beginning of the recording period, plants of genotype F5 were significantly taller than those of the other two genotypes (Tukey's unequal N HSD, S11: $p<0.003$; 47: $p<0.0003$). However, by the end of the recording period in September 2004, plants of genotype S11 were significantly taller than those of the other two genotypes (Tukey's unequal N HSD, F5: $p<0.0004$; 47: $p<0.0001$).

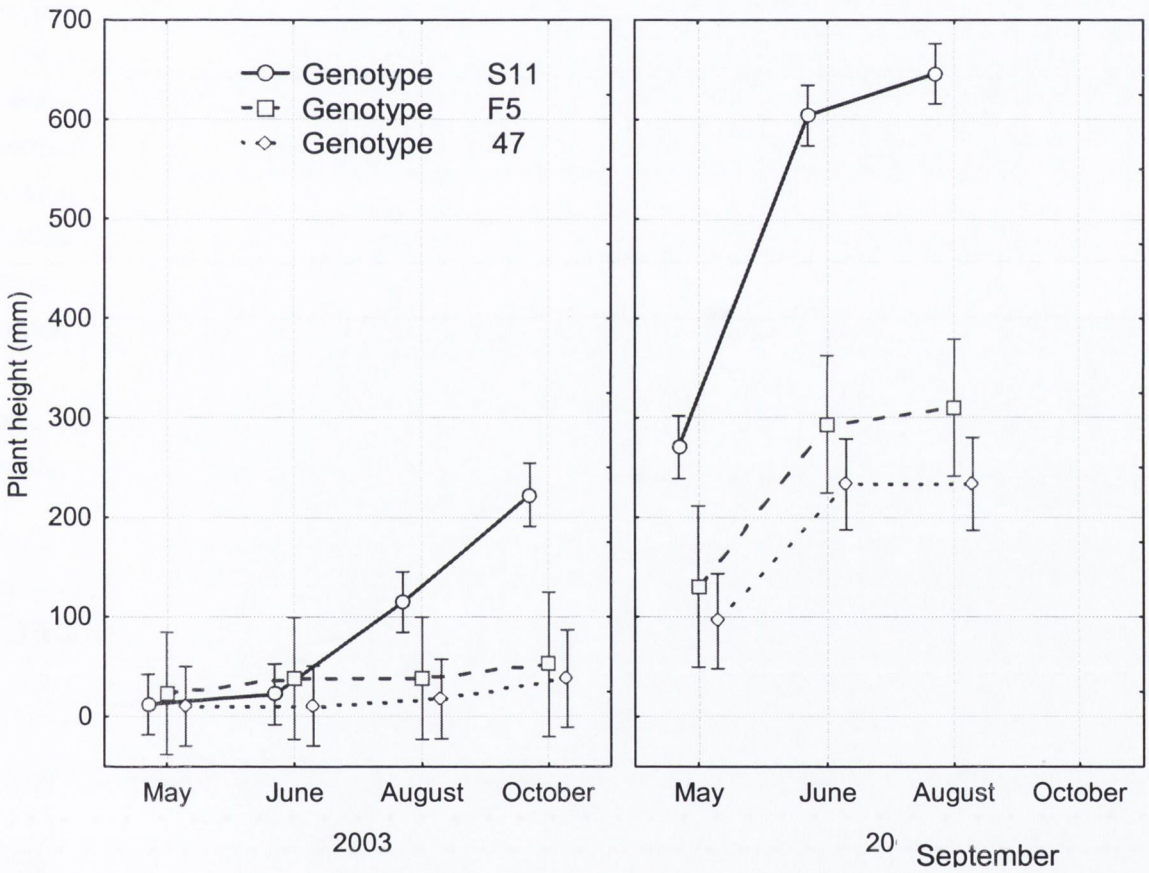


Figure 5. 4. The growth of micropropagated plants of three genotypes over two consecutive years after weaning.

(15) *Root induction experiments on dormant cuttings from previously micropropagated plants and from grafted plants.*

Root induction treatments were administered to dormant cuttings taken from grafted plants growing in field conditions and from micropropagated plants growing in the greenhouse at Teagasc Kinsealy Research Centre (section 5. 5. H. (15)). The results are shown in table 5. 25. and 5. 26. Only 1 cutting from the dormant grafted plants rooted (genotype 72 treatment 1), and very few produced callus (11%). It was noted that many of the cuttings from grafted plants had rotting stem bases and dead wilted shoots at the time of recording.

Cuttings from the micropropagated plants responded well to the treatments and it was noted that most of the shoots appeared healthy and were growing well, even those in the control treatments were producing roots. There was very little callus production (11%) from the shoots. A significantly higher proportion of nodal cuttings rooted (40.0%) compared to apices (31.6%) and there was a significant difference in rooting between genotypes (Chi square test, $p<0.04$ and $p<00001$, respectively). Genotype F5 had the least number of shoots rooting (6.2%) followed by genotype 72 (37.5%), genotype 47 (50.6%) and genotype 49 (57.5%). There was no significant interaction in rooting percentage between genotype and cutting type, genotype and treatment or cutting type and treatment, all genotypes and cutting types responded similarly to the treatments tested (Factorial ANOVA, $p=0.11$, $p=20$ and $p=0.51$). Rooting was significantly lower compared to all other treatments in cuttings from treatment 5 (Tukey's unequal N HSD, $p<0.02$). There was no significant difference between treatments for the number of roots produced per rooted cutting (One-way ANOVA, $p=0.06$, mean value 4.1 ± 2.3).

Table 5. 25. The effect of rooting treatment, cutting type (apical or nodal) on rooting in cuttings of four genotypes derived from dormant grafted plants.

	Genotype					
	47		*49*		*72*	
Treatment *	Apical cutting	Nodal cutting	Apical cutting	Nodal cutting	Apical cutting	Nodal cutting
1	0	0	0	0	5	-
2	0	0	0	0	0	-
3	0	-	0	-	0	-
4	-	0	-	0	-	-
5	0	0	0	0	0	-

* 1= control; 2= Seradix® powder (contains 8000ppm IBA) applied to base; 3= 8000ppm IBA in lanolin applied to the apical bud; 4= 8000ppm IBA in lanolin applied to the apical cut surface; 5= 8000ppm IBA in lanolin applied to the cutting base.

Table 5. 26. The effect of rooting treatment, cutting type (apical or nodal) on rooting in cuttings of four genotypes derived from dormant micropropagated plants.

	Genotype								
	F5		*47*		*49*		*72*		*Total* *
Treatment**	Apical cutting	Nodal cutting	Apical cutting	Nodal cutting	Apical cutting	Nodal cutting	Apical cutting	Nodal cutting	
1	0	30	25	65	60	-	30	45	36.4^{a***}
2	0	5	45	70	70	-	40	60	41.4^{a}
3	0	-	60	-	65	-	45	-	42.5^{a}
4	-	10	-	75	-	-	-	50	45.0^{a}
5	5	0	15	50	35	-	10	20	19.3^{b}

* Data combined for all cutting types and genotypes. ** 1= control; 2= Seradix® powder (contains 8000ppm IBA) applied to base; 3= 8000ppm IBA in lanolin applied to the apical bud; 4= 8000ppm IBA in lanolin applied to the apical cut surface; 5= 8000ppm IBA in lanolin applied to the cutting base. *** Different letters between rows indicate a significant difference according to Tukey's unequal N HSD, $p<0.02$.

5. 7. Discussion

In this chapter, treatments for inducing root growth from *in vitro* propagated shoots of *F. excelsior* from a range of genotypes were elucidated and the progress of plantlets weaned to the glasshouse was monitored. Furthermore, it was demonstrated that dormant cuttings from plants produced by the micropropagation cycle are capable of rooting under plastic in the greenhouse.

5. 7. 1. IBA exposure time

A pulse treatment for 2-6 days on media containing IBA, followed by transfer to media with no IBA containing activated charcoal gave optimum results for rooting shoots compared to treatment with continuous exposure to IBA. For example, in experiment 4 (section 5. 6) shoots of genotype S8 and 47 failed to root during continuous IBA exposure whereas in pulse exposure treatments achieved a maximum of 10% and 50% rooting respectively. In the pulse exposure treatment rooting of the mature genotype tested (genotypes 47) increased with IBA concentration (tables 5. 7 and 5. 9) whereas in the continuous supply treatment applied in experiment 1 (section 5. 6) (where shoots of genotype S11 and 49 did root) rooting decreased as IBA concentration increased (table 5. 3). The optimum level of auxin required to promote rooting in the pulse exposure treatment varied with genotype, the mature genotype having highest rooting percentage in medium with 20mg/l IBA. IBA concentration in the medium had no significant effect on rooting in the seedling genotypes (S8 and S11). It was also noted that callus formation on the bases of the shoots was most frequent in the shoots continuously supplied with IBA. Hammatt and Ridout (1992) rooted shoots of *F. excelsior* by using a pulse treatment on IBA containing medium for three weeks followed by transfer to medium without IBA. Similarly, it was found that rooting of the shoots increased with IBA concentration. Silveira and Cottignies (1994) used no auxin in their root induction media for *F. excelsior*. Other *Fraxinus* spp. have been shown to root equally well without any auxin treatment (Bates *et al.*, 1992; Tonon *et al.*,

2001), and those that use auxin continuously in the rooting medium tend to do so at very low concentrations (0-1mg/l) (Chalupa, 1990; Perez-Parron *et al.*, 1994). Several authors have shown that auxin is only required during the root initiation phase and becomes inhibitory for root outgrowth (Haissig, 1970; De Klerk *et al.*, 1990). An inhibitory effect of auxin is also observed when explants are exposed to a too high concentration of IBA, and is often accompanied with callus formation (Pawlicki and Welander, 1995). Previous studies have supported the possibility that activated charcoal absorbs toxic substances released by the explants (Klein and Bopp, 1971) and promotes root formation (Pierik, 1987b). Proskauer and Berman (1970) studied the influence of rooting medium illumination on root induction using activated charcoal in order to cause a total or partial darkening of the medium. In root induction experiments with *Quercus robur* and *Q. rubra* L. Sanchez *et al.* (1996) concluded that the concentration and exposure time of the IBA treatment were critical for root induction. In both *Quercus* species, best rooting efficiency was achieved by culture in medium containing 25 mg/l IBA for 24 h and subsequent transfer to an auxin-free medium containing 1% activated charcoal. For all clones tested, the charcoal benefited both shoot quality and root system development, the latter being enhanced by the formation of many lateral roots (Sanchez *et al.*, 1996).

5. 7. 2. The influence of light on rooting

For both the pulse and continuous exposure to IBA treatments in this thesis, rooting percentage was increased by placing the shoots in darkness for 2-8 days (table 5. 5 and 5. 6). Artificially darkening the media with a foil covering, black dye or activated charcoal increased the number of shoots rooting in seedling genotype S8 however, in mature genotype 49 rooting was only increased by the charcoal treatment and was decreased, compared to the control, by the foil covering and the black dye darkening treatments (figure 5. 3). For the seedling genotype S8 adding black dye to the media best promoted rooting (45%), whereas for the mature genotype 49 adding activated charcoal best promoted rooting (87%) (table 5. 12). It is well known that darkness during the first week of the rooting phase is often essential in stimulating rooting in several woody species (Druart *et al.*, 1982; Sriskandarajah *et al.*, 1982; Ruigini and Verma, 1983; Ruigini *et al.*, 1993; Pawlicki and Welander, 1995; Pierik and Sprenkels, 1997; Mencuccini, 2002). Pierik and Sprenkels (1997) showed that a four day dark period after the start of root initiation was required to give high rates of rooting in mature *F. excelsior*. Darkening only the basal part of olive, almond, chestnut and walnut explants during the rooting phase by painting the outside of the jar black and covering the medium surface with black polycarbonate granules, induced rooting and was essential for root induction in chestnut, walnut and almond (Ruigini *et al.*, 1993). Sanchez *et al.* (1996), in studies involving *Quercus robur* and *Q. rubra,* concluded that because darkening the basal part of the shoots with aluminium foil during the rooting phase only caused a small increase in rooting, the large effect of charcoal on rooting was the result of adsorption of inhibitory compounds from the medium or the explant or both, rather than of basal darkening. This may explain the large increase in rooting percentage for genotype 49 in the activated charcoal darkening treatment. In previous experiments, genotype 49 was inhibited from rooting by the continuous supply of IBA in the rooting medium (table 5. 3 and section 5. 6 (4)), the activated charcoal added to darken the medium may have absorbed the inhibitory IBA contained in the medium. Rooting in genotype S8 appeared not to be affected by the continuous supply of IBA in the medium, therefore the activated charcoal had no effect on root induction for this genotype. Darkening the media with black dye guarantees the basal darkening of explants (Mencuccini, 2002) and is more practical than the painting of vessels as reported by Ruigini *et al.* (1993).

Mencuccini (2002) reported that basal shoot darkening by adding black dye (100mg/l) to the media promoted rooting in *Olea europaea* and even overcame seasonal differences in rooting ability. It is known that auxin transport is affected in a light-dependent manner (Jensen *et al.*, 1998), which has been implicated in playing a role in the shade avoidance response (Morelli and Ruberti, 2000); the phytochrome-mediated phosphorylation of Aux/IAA proteins has been suggested as a mechanism for integrating auxin and light signals in plant development (Colón-Carmona *et al.*, 2000). The results of this thesis showed a possible toxic effect of the dye in genotype 49, in which rooting was reduced to 8%. This was not seen for genotype S8 for which the dye promoted rooting (45%).

5. 7. 3. The influence of cytokinin on rooting

Adding 1mg/l BAP to the rooting media slightly increased the number of shoots rooting (tables 5. 14 to 5. 16). However, differences were small in comparison to other manipulated factors such as IBA exposure time and light. In many plant species it has been shown that optimal root formation occurred in the presence of auxins and cytokinins (Gamborg *et al.*, 1974, Chlyah and Tran Thanh Van, 1975). Arrillaga *et al* (1992) found that adding BAP to the rooting medium only increased rooting in *F. ornus* when in combination with NAA but had no effect when added with IAA or IBA. Optimal rooting in *Prunus cerasus* was obtained with both BAP and IBA in the medium (Poncia and Roselli, 1980). These observations suggest that an optimal endogenous auxin-cytokinin balance might have been established for root formation (Nemeth, 1986). Although, cytokinins are considered to inhibit rooting (Varga and Humphries, 1974), at later stages, the inhibitory effect of cytokinins disappear and development of root primordial seems to depend on cytokinins (Eriksen, 1974).

5. 7. 4. The influence of media salts on rooting

Of the four media salt solutions tested, shoots placed on medium containing modified WPM salts had the highest rooting percentage (table 5. 18). The suitability of WPM as a rooting medium for *Fraxinus excelsior* has been confirmed in this study and in previous studies including Preece *et al.*, 1987, Chalupa, 1990; Hammatt and Ridout, 1992; Silveira and Cottignies, 1994. Pierik and Sprenkels (1997) used 0.1 strength MS macrosalts in the root induction medium for *F. excelsior*. Mokotedi *et al.* (2000) found that rooting increased and callus formation decreased as the nutrient concentration of rooting medium for *Eucalyptus grandis* Hill ex Maid. X *E. nitens* (Deane & Maid.) Maid. was reduced from full strength MS to ¼ strength. Furthermore, the roots obtained in the lower strength media were longer and thicker than those produced in the high strength media. The promotory effect of mineral concentration on rooting can be explained by considering that the inorganic ions participate in processes regulating hormonal balance (Amzallag *et al*, 1992). Calcium ions in particular (which are in higher concentration in modified WPM and QRC media compared to MS) are essential for the basipetal transport of endogenous IAA in both roots and shoots, and may also have important regulatory effects on the cell elongation process and gravity perception in plants (Kaufman *et al.*, 1995). Kaira and Bhatla (1998) considered that a net influx of extracellular calcium in the differentiating cells accompanies the auxin-induced response during rooting. When calcium channel blockers were added to root induction medium for *Helianthus annuus* L. rooting was inhibited (Kaira and Bhatla, 1998).

5. 7. 5. The influence of explant type on rooting

Both shoot tip and nodal explants were equally capable of producing roots *in vitro,* and showed a similar response to IBA treatment (section 5. 6 (1)). Marks (1996) showed that root induction in *Quercus robur* and *Daphne cneorum* depended on the presence of a growing apex and that rhizogenesis in woody species *in vitro* was mediated through the basipetal transport of auxin from the shoot apex. However, shoots of *Betula pendula* rooted easily with or without an apex suggesting that either a factor produced in the apex is essential for rhizogenesis to occur in *Q. robur* and *D. cneorum* but not in *B. pendula* (or in the ash studied here) or that endogenous auxin can be supplied from elsewhere such as a quiescent axillary bud. That basipetal transport of auxin modulates rhizogenesis was demonstrated by the inhibition of root induction in *Betula* shoots by the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA), and by the substitution of IAA for a bud in *Betula* internodal sections. Ruigini *et al.* (1993) achieved rooting successfully from both shoot tips and nodes of *O. europaea*. Without any induction treatment the shoot tips rooted in higher percentage than the nodes. However, it was found that treatment with basal shoot darkening or putrescine increased rooting in nodal shoots but had no effect on shoots tips.

5. 7. 6. The influence of shoot proliferation medium on rooting

There was no significant difference in rooting capacity between shoots taken from M91 medium (80%) or from QRC media with charcoal (68.6%) (table 5. 17). Many authors working with *Fraxinus* species report taking shoots for root induction treatments directly from the shoot production media (Chalupa, 1990; Hammatt and Ridout, 1992; Silveira and Cottignies, 1994 and Perez-Parron *et al*, 1994). Such results were supported in this study. However, a carry over effect of hormones in the shoot multiplication medium, on rooting responses, has been reported in other species including *Eucalyptus globulus* Labill. and *Quercus robur* (Bennett *et al.*, 1994; San-José *et al.*, 1988). Bennett *et al.* (1994) observed that in *Eucalyptus globulus* shoots taken from multiplication medium with BAP produced few roots and often died. In *Quercus robur* rooting was greatly improved when shoots are transferred to an elongation medium prior to root induction (San-José *et al.*, 1988). An important component of successful root induction for such species includes the use of a conditioning medium, prior to rooting. Conditioning media may include low levels of gibberellins (Durand-Cresswell *et al.*, 1982; Lakshmi and Rhani, 1985), auxin (McComb and Bennett, 1986), phenolic compounds (Zimmerman, 1984), and cytokinins (Curir *et al.*, 1990) to stimulate elongation of the shoots prior to root induction. This further supports the notion that certain species such as *Q. robur* may require the shoot apex to be actively growing for rhizogenesis to occur.

5. 7. 7. The effect of harvesting shoots at different times after transfer on rooting

Taking shoots at different times after transfer to plant growth regulator free QRC medium with charcoal had no significant effect on rooting (tables 5. 21 and 5. 22). Similarly, as for the previous experiments, it was noted that variations for rooting capacity existed between different genotypes of *F. excelsior* and within the same genotype at different times. Some possible explanations for the variation in rooting capacity are outlined. Root formation *in vitro* depends, not only on medium composition and treatments but also on many endogenous factors such as genetic background, physiological influences, age and the ontogenetic phase of the mother plant (Németh, 1986). Attempts have been made to understand the mechanisms that control rooting: some authors have compared the *in vitro* rooting performance of cultivars, or clones or various

genotypes with high and low rooting capacities (Collet *et al.*, 1994; Baraldi *et al.*, 1995; Jay-Allemand *et al.*, 1995), while others (Berthon *et al.*, 1989; Hausman, 1993; García-Gómez *et al.*, 1994; Heloir *et al.*, 1996) have applied root inductive stimuli to a single genotype at a specific developmental stage. However, only in a few cases was the inherent rooting capacity related to the different ontogenic stages of the same genotype (Geneve *et al.*, 1988; Sánchez and Vieitez, 1991; Murray *et al.*, 1994; Jay-Allemand *et al.*, 1995; Diaz-Sala *et al.*, 1996). Puente and Marín (1997) concluded that differences in the rooting behaviour of an individual *Malus* X *domestica* Borkh. clone were due to changes in the short term physiological state of the shoots. Ballester *et al.* (1999) studied *in vitro* rooting in *Castanea sativa* shoots of the same genotype displaying juvenile (easy to root) and mature (difficult to root) characteristics. Culture lines collected from the base (juvenile) or crown (mature) of an 80 year old tree, both responded differently to root induction treatment showing that even after seven years of *in vitro* culture the culture line "remembered" its original position on the tree. The cells of shoots with juvenile character initiated division in response to auxin treatment and later developed root primordia whereas, although, the cells from mature shoots initiated cell division, normal cambial derivatives were produced instead of root primordia. Furthermore, the rooting response of shoots to IBA treatment was found not to be a function of the endogenous auxin level. The results of Marks (1996) point to an interaction between endogenous auxin affecting the competence of tissues to respond, and to the role of exogenous auxin in providing a signal to initiate root induction. Differential gene expression in juvenile and mature phases may be directly related to the rooting process (Ballester *et al.*, 1999). A gene which codes for a cell wall proline-rich protein was more highly expressed in mature than in juvenile petioles of *Hedera helix* (Woo, Hackett and Das, 1994), and differences in the expression of actin, a cyclin dependant kinase, and expansin, a gene which causes non-hydrolytic cell wall loosening, were observed between hypocotyls (easy to root) and epicotyls (difficult to root) of *Pinus taeda* L. (Greenwood *et al.*, 1997).

5. 7. 8. Weaning

Plants that were rooted *in vitro* had better survival rates (80.6%) when compared to plants that were transferred to *ex vitro* weaning conditions without roots (24.7%) (table 5. 23). For some easy-to-root plants, it is more convenient and less labour intensive to carry out the rooting process on *in vitro* produced microshoots *ex vitro* (Bates *et al.*, 1992; Kim *et al.*, 1997). However, most authors working with *Fraxinus* species report only transplanting rooted plantlets to the *ex vitro* environment and there are few reports of any difficulties establishing rooted plants in the soil (Chalupa, 1990; Arrillaga *et al.*, 1992; Hammatt and Ridout, 1992; Hammatt, 1994; Silveira and Cottignies, 1994; Tonon *et al.*, 2001). The "unrooted" plants that survived may have already formed root initials which were not visible at the time of planting but developed when transferred to the soil medium in the glasshouse.

Weaned plants that received a cold period over winter resumed growth earlier in the season compared to weaned plants that were kept over winter under artificial heat and light in the glasshouse (table 5. 24). In both treatments the plants appeared to become endodormant (discussed in chapter 2). Plants in the endodormant state can emerge from it only after a sufficient chilling period (Saure, 1985). However, when the endodormancy chilling requirements are fulfilled the plants may remain in a lesser state of dormancy (ecodormancy) until environmentally favourable conditions return (Horvath, 2003). Plants over wintered in the glasshouse appeared dormant in so far as there was no shoot growth and they had a terminal resting

bud. However, because they did not receive a chilling treatment the endodormancy requirements were not fulfilled and they took longer to resume growth in the spring. This response has previously been shown in deciduous fruit trees grown in the tropics where insufficient chilling resulted in an extension of the endodormant period and a delay in bud break (Saure, 1985). The results described in table 5. 24. and figure 5. 4. show that the chilling requirement is genetically determined (100% of plants of genotype 49 were still dormant at time of recording whereas 65% of plants from genotype S11 were in active growth, table 5. 24). Chalupa (1990) planted weaned *F. excelsior* trees in the field after two months hardening in the greenhouse and reported that the plants survived well over winter and grew normally with good clonal uniformity.

Tissue-culture plantlets weaned to the glasshouse during winter 2003 grew poorly during the first year of growth (figure 5. 4). In the first season, plants of the mature genotypes F5 and 47 hardly grew at all from May to October 2003, whereas plants of the seedling genotype S11 had begun growing after June 2003 and continued to do so until the final measurement in October 2003. In the second year, plants of all three genotypes had begun growing by the first measurement period in May 2004 and had gained considerable height by the final measurement in September 2004. The delay of growth during the first season may be explained by the plants not receiving a winter chilling period. The chilling requirement of trees is known to be genetically determined (Saure, 1985) and may account for the resumption of growth of genotype S11 while the other two genotypes remained relatively dormant. In 2004, the plants received chilling over winter as the glasshouse they were kept in had no artificial heat or light, and as a result growth resumed normally in all three genotypes. This further highlights the importance of fulfilling the chilling requirements of the trees for normal growth to occur. Within the genotypes all the plants were relatively uniform and both mature genotypes 47 and F5 had a strong leading shoot with no plagiotropic growth observed. Hammatt (1997) reported that trees of a micropropagated clone had reached a mean height of 806mm after a three year period, in this chapter, trees of the three micropropagated genotypes S11, F5 and 47 reached mean heights of 646mm, 310mm and 233mm respectively, after two years growth.

5. 7. 9. Rooting dormant cuttings

Dormant cuttings taken from plants that had been though the micropropagation cycle were induced to produce roots whereas similar dormant cuttings from field grown grafted plants failed to root. As seen in previous rooting experiments, the rooting capacity of cuttings and their response to root induction treatments varied between genotypes. In general, treatments with IBA did not significantly improve rooting compared to cuttings receiving no auxin treatment (table 5. 26). In the control, the proportion of nodal cuttings rooting (30-65%) was higher than the apical cuttings (0-60%). Applying IBA in the form of Seradix® powder increased rooting in all cutting types and genotypes (45-70%), except genotype F5 for which rooting was decreased (5%). Applying IBA mixed with lanolin to the apical bud of cuttings increased rooting in most genotypes (45-60%, genotype F5: 0%). The same treatment applied to the proximal cut end of the nodal cuttings increased rooting only marginally (50-75%) and in genotype F5 decreased the rooting capacity of cuttings (10%). Applying IBA in lanolin to the base of both cuttings types decreased rooting (5-50%) compared to the controls (30-65%).

According to George (1993) woody plants derived from micropropagation may retain the effects of rejuvenation *in vitro* when they are grown and propagated conventionally, an increased rooting capacity is frequently used as an indicator of rejuvenation or reinvigoration (Gupta *et al.*, 1981; Lyrene, 1981; Sriskandarajah *et al.*, 1982). Nickerson (1995) used tissue-cultured *Syringa vulgaris* liners for propagation from soft wood cuttings and found the most important factor affecting rootability to be the juvenility of the parent plant from which the cutting was made. The level of dormancy, vigour and juvenility seem to be major factors in rooting (Howard, 1981; Kunneman and Otten, 1994; Kunneman, 1995). Silveira and Cottignies (1994) reported "limited root induction" in nodal cuttings of juvenile *F. excelsior* trees during the dormant period whereas cuttings taken during the growing period failed to root. In this case, the stem cuttings were heat treated to break dormancy and then placed in trays in the greenhouse, no auxin treatment was applied (Silveira and Cottignies, 1994). Richardson (1958) observed that the completion of bud dormancy and the initiation of new root growth are closely connected in *Acer saccharinum*, and that root growth is only possible when at least one bud is physiologically non dormant. In the present study the nodal cuttings, which were released from their dormancy, were more likely to root. This may have been due to increased endogenous auxin production from the outgrowth of two nodal buds. Young and Westwood (1975) and Erez and Yablowitz (1981) were also able to establish unequivocally a relation between bud activity and inhibition or stimulation of rooting. The decreased rooting in cuttings with IBA applied in lanolin compared to IBA in talc (in the form of Seradix® powder) may be due to the increased absorption of IBA when applied with lanolin. The increased absorption of IBA by the cells on the stem base resulted in an extended period of exposure to high IBA levels. Such continuous exposure to IBA was previously shown to inhibit rooting.

Rooting responses vary greatly within and between species with many shoots forming roots rapidly while others are much slower. For many species, the controls which direct adventitious rooting *in vitro* remains obscure and one of the most difficult areas of micropropagation research (Bunn and Dixon, 1996). This thesis has shown that auxin has a significant effect on rooting in ash and according to Blakesley (1994) seem to be the main class of regulatory molecules acting on the rooting process. According to De Klerk *et al.* (1997) and as shown in this thesis for ash, auxins are required for rooting during the induction phase, but can inhibit the process during the formation phase.

The experimental work in this chapter has shown that the optimum treatment for root induction from shoots of *F. excelsior* produced in the shoot production phase of micropropagation is a pulse treatment on auxin containing media in darkness, followed by transfer to auxin free medium containing activated charcoal and subsequent culture in the light. This treatment has proven to be effective in a range of genotypes and is used as standard in the laboratory in Teagasc, Kinsealy. The rooted plantlets were successfully weaned to the glasshouse and can survive outdoors over winter, in fact benefiting from the chilling received by resuming growth earlier than plantlets kept over winter in a heated glasshouse. It has also been demonstrated that dormant winter cuttings taken from tissue-culture produced plants can be induced to form adventitious roots perhaps indicating that reinvigoration or rejuvenation of mature tissues has taken place *in vitro.* It has now been shown that tissue-cultured trees of mature genotypes can be used to produce cuttings of both summer shoots (Douglas, personal communication) and dormant winter shoots for root induction (this chapter).

CHAPTER 6: Analysis of the genetic fidelity of vegetative propagules and the genetic diversity of a sample of the selected population using molecular markers.

6. 1. Introduction

The three basic phases of a tree improvement program include conservation, breeding, and propagation. An important strategy in tree improvement is to make use of the genetic diversity of the natural population and to ensure that it is well represented in the selected trees. The most direct way to capture the special genetic makeup of a selected, mature tree is through mass vegetative propagation of that particular tree (chapters 3, 4 and 5). However, it is characteristic of most woody species including ash that they resist traditional vegetative propagation methods such as the rooting of cuttings after the mature phase of growth of source trees is well underway (Cahalan and Jinks, 1992; chapter 5). Grafting shoots of selected mature trees onto seedling rootstock has been a successful method to vegetatively propagate ash trees but is labour intensive and if trees are not regularly checked, shoots from the rootstock can outgrow the grafted scion making it difficult to discern the true origins of the tree (Douglas, personal communication). Tissue culture propagation methods offer a way round this obstacle and the promise of larger numbers of propagules in a shorter time than could be obtained with rooted cuttings or grafting (Mott, 1986).

Micropropagation methods using apical and axillary buds as primary explants have been developed for selected mature trees of *F. excelsior* (Hammatt, 1994; Silveira and Cottignies, 1994; Hammatt, 1997; Pierik and Sprenkels, 1997; chapters 3 and 4), however plantlets derived from *in vitro* culture may exhibit somaclonal variation which is often heritable and therefore results in stable genetic changes (Karp, 1991; Vendrame *et al.*, 1999; Vendrame *et al.*, 2000; Carolan *et al.*, 2002). Hence, it is necessary to control the genetic integrity of the *in vitro* propagated plants. This chapter aimed to assess the genetic fidelity of vegetatively propagated trees of *F. excelsior* by microsatellite (SSR) analysis of nuclear DNA. DNA was extracted from grafted plants of the selected trees conserved by Coillte in Kilmacurra, Co. Wicklow and in Teagasc, Kinsealy and from micropropagated plants in the shoot production phase of *in vitro* culture at the laboratory at Teagasc, Kinsealy and those weaned to the glasshouse. Furthermore, the level of genetic diversity in a sample of the selected trees was elucidated and compared to data from studies by Harbourne (2004) which assessed the genetic diversity in the natural populations and in trees from an Irish provenance trial of ash. The thesis of Harbourne (2004) examined biogeographical and population genetic variation of ash in Ireland and other European countries, it therefore provided an excellent source of data for comparison.

6. 2. Microsatellite markers and the assessment of diversity

Within the past decade microsatellite markers have developed into one of the most popular genetic markers. Their popularity stems from their near ubiquity, general high level of polymorphism, codominance, and relative ease of screening once isolated (Van Oosterhout, 2004). SSR technology has been widely used in the assessment of botanical diversity and somaclonal variation (discussed chapter 1 section 1. 8 and 1.9). Microsatellite markers for use in analyzing the genome of *F. excelsior* were first developed in 1999 (Brachet *et al.*, 1999; Lefort *et al.*, 1999) and have since been successfully employed to assess the diversity of many ash populations (Heuertz *et al.*, 2001; Morand *et al.*, 2002; Heuertz *et al.*, 2003; Harbourne, 2004). They have also been used in hybridization studies between *F. excelsior* and *F. angustifolia* (Racquin *et al.*, 2002). The microsatellite loci used by previous researchers contained polymorphic alleles allowing clear distinction between individual genotypes. In particular, the loci named M2-30, FEMSATL 4, 11, 16 and 19 used by Heuertz *et al.,* (2001) and Heuertz *et al.* (2003) appear to be highly polymorphic and in these two studies, on ash populations in Bulgaria and Romania, produced between 10 and 59 alleles at each locus.

Understanding the mutational process of microsatellite DNA sequences is essential before relationships between observed variation and genetic distance or population substructure can be inferred. Microsatellite DNA sequences mutate at rates several orders of magnitude higher than that of the bulk of DNA (Ellegren, 2000a). Such high rates mean that spontaneous mutations that form new-length variants can realistically be seen in closely related plants of the same species. According to Ellegren (2000a) a striking feature of the mutation process is that it is highly heterogeneous, with distinct differences between species, repeat types, loci and alleles. Age and sex can also affect the mutation rate. For DNA sequences evolving neutrally, such as those at most microsatellite loci, the amount of polymorphism is expected to be directly proportional to the underlying mutation rate (Kimura, 1983). Hence, it has been assumed that the mutation rate to form new-length variants at microsatellite loci is appreciable (Weber and Wong, 1993; Goldstein and Pollock, 1997).

A mutation event is typically recognized as a shift in allelic mobility during electrophoretic separation when comparing amplification products (via PCR) of different plants. Misinterpretation of results can arise from the existence of null alleles (alleles that are never amplified because of non-matching primer sequences), which can be suspected for microsatellite loci, especially when using heterologous primers (Pemberton *et al.*, 1995). Morand *et al.*, (2002) calculated the frequency of null alleles at five *F. excelsior* microsatellite loci as between 0.25 and 0.38. Allelic dropout (the failure to amplify one of an individual's two alleles) can be a serious problem in low quantity DNA samples and is caused by stochastic sampling error (Navidi *et al.*, 1992; Taberlet *et al.*, 1996). If template DNA is at very low concentrations, then one copy may, by chance be amplified more than the other. If the two copies

represent different alleles (a heterozygote) then dropout yields a false homozygote. Taberlet *et al.*, (1996) estimated that at low concentrations of DNA the rate of allelic dropout can be greater than 50%. Polymerase error rendering a false allele and amplification of contaminant DNA are genotyping errors that occur less frequently at microsatellite loci. Contamination can be controlled by stringent laboratory protocols and the inclusion of negative controls (Miller *et al.*, 2002). False alleles are considerably less frequent (Gagneux *et al.*, 1997; Goossens *et al.*, 1998), and often show an unusual spectral pattern, or produce three alleles that can be tagged as suspicious. Nevertheless, cryptic false and contaminant alleles do occur and when they are undetected, they will cause genotype errors. The current practice of scoring microsatellites using electropherograms (from automated sequencers) provides much greater resolution for identifying anomalous patterns compared to the former technique of scoring alleles by autoradiography (Miller *et al.*, 2002).

6. 2. 1. Mutation mechanisms of microsatellite loci

The mutation model based on replication slippage formulated by Levinson and Gutman (1987) appears to be the predominant mode of mutation at microsatellite regions (Wolff *et al.*, 1989). Replication slippage or slipped-strand mispairing refers to the out-of-register alignment of two DNA strands following dissociation of a polymerase complex. This slippage creates a transient bulge which upon DNA repair would be either removed or lead to the elongation or shortening of the repeat (Schlotterer and Tautz, 1992). The possible existence of other mutation mechanisms contributing to length variation at microsatellite loci is a long-standing debate in the microsatellite literature. In theory, new length variants at repetitive DNA sequences can form through interchromosomal exchange, for example with recombination (unequal crossing-over) (Hu and Worton, 1992) or gene conversion. Gene conversion is the nonreciprocal transfer of genetic information, if the sequence used as a template for repairing a gene by homologous recombination differs slightly from the gene needing repair; that is, is an allele, the repaired gene will acquire the donor sequence.

The stepwise mutation model of Ohta and Kimura (1973) stating that alleles can only mutate by the gain and loss of one repeat unit was proposed by Valdes *et al.* (1993) to explain the distribution of allele size at 108 microsatellite loci. A more detailed study by Shriver *et al* (1993) using four summary measures of allele frequency and data from large homogenous populations concluded that the VNTR loci, including microsatellites, have a mutational mechanism very close to but not exactly like the one-step model. Of the hypothesized mechanisms of VNTR mutation, replication slippage is most like the stepwise mutation model and so is the most likely mechanism of mutation (Li, 1997). Moreover, *in vitro* experiments clearly demonstrate that microsatellite sequences have the intrinsic ability to undergo DNA slippage (Schlötterer and Tautz, 1992).

6. 2. 2. The mutation rate of microsatellite loci

The microsatellite mutation rate is estimated to fall within the range of 10^{-2} to 10^{-3} per locus per generation for nuclear-encoded loci in plants (Kovalchuk *et al.*, 2000a; Udupa and Baum, 2001). One major determinant of the variation in mutation rates between loci is the length of the individual microsatellite. For most species studied the average repeat number is directly proportional to the degree of length polymorphism, indicating that long loci mutate more often than short loci (Weber, 1990). This view has now been extended and supported by direct estimates of mutation rates at different loci in the human genome (Brinkmann *et al.*, 1998; Kayser *et al.*, 2000). Importantly, the dependence of mutation rate on repeat length further implies that rate variation might extend to variation within loci that is, between alleles (Ellegren, 2000a). Thus, particularly high mutation rates have been discerned at long alleles of individual microsatellite loci in for example, birds (Primmer *et al.*, 1996, 1998), *Drosophila melanogaster* (Schlötterer *et al.*, 1998) and ants (Crozier *et al.*, 1999). Moreover, pooled data from mutations at human di- and tetra-nucleotide loci showed a positive correlation between standardized allele size and incidence of mutation (Ellegren, 2000b). It is plausible that the positive correlation between mutation rate and allele size can be explained by the fact that replication slippage can take place at more locations in a repetitive sequence with many repeat units (Ellegren, 2000a). Another factor proposed to affect the microsatellite mutation rate is the size difference between an individual's two alleles, the "allele span" (Amos *et al.*, 1996). However, this idea has an inherent problem given the evidence for mutations representing intrahelical events, and is not supported by recent data (Ellegren, 2000b). It has also been speculated that the flanking sequence context might affect the mutation rate, for example, through a simple GC content effect or by *cis*-acting modifiers (Glenn *et al.*, 1996; Brock *et al.*, 1999). There is, however, no strong empirical data to support this idea for microsatellites in general (Balloux *et al.*, 1998).

The vast majority of microsatellite mutations apparently represent gains or losses of entire repeat units, a hallmark of replication slippage (Ellegren, 2000a). In the classical stepwise mutation model, the probabilities of addition and deletion of a single repeat unit are identical and constant across the alleles however, as discussed in the previous paragraph, mutation rates are not constant across different allele sizes. Similarly, evidence is accumulating to suggest that other assumptions from the classical stepwise mutation model are violated. First, mutations do not always constitute single step changes, although this seems to be the most common type of mutation. Second, a marked mutational bias in favour of expansions over contractions is shown in studies using an avian tetranucleotide locus (Primmer *et al.*, 1996, 1998). These are important observations because they show that the evolutionary process leading to length variability at microsatellite loci do not follow a simple mutation model, in particular not a strict stepwise model (Ellegren, 2000a).

6. 3. Genetic heterogeneity within organisms

Individuality plays a central role in ecology and evolution where concepts such as population, birth, reproduction, and death are defined at the level of the individual organism. In spite of this, the concept of individuality is lacking a clear definition and used ambiguously in the literature. An individual is traditionally defined as reproductive, physiologically united, autonomous, genetically homogeneous and unique entity, which is also the main unit of selection (Tuomi & Vuorisalo, 1989; Fagerström *et al.*, 1998; Michod, 1999; Santelices, 1999). The invariability of the second two attributes over the years has been disproved for many organism groups. Physiological unity and autonomy is absent in social colonies and genetic uniqueness is lacking in clonally derived organisms (Santelices, 1999). Genetic homogeneity is still, however, commonly assumed to hold true. However, studies have accumulated where naturally occurring intraorganismal genetic heterogeneity (IGH) has been documented from all branches of life suggesting that it is much more common than previously believed (Pineda-Krch and Lehtila, 2004). The majority of knowledge on the prevalence of naturally occurring IGH and its effects mainly come from the fields of horticulture, animal husbandry and medicine.

There are two main types of IGH, mosaicism and chimerism, which refer to the functional origin of the heterogeneity. In a mosaic individual the IGH originates from various forms of genetic change, e.g. somatic mutations, mitotic recombination, mitotic gene conversion (Otto & Hastings, 1998; Youssoufian & Pyeritz, 2002), genome duplications, and changes in ploidy level (Santelices, 1999). Chimeric individuals, in contrast, originate through a fusion process, either by fusion of two individuals or exchange of genetically distinct parts which can be single-celled or multicellular units. Formally a chimera is defined as the presence of two or more cell lines that are derived from different zygotes. Normally, cellular self-recognition systems prevent fusion with all nonself cells except close kin (Buss, 1982; Grosberg & Strathmann, 1998). Thus, it seems likely that the majority of genetically heterogeneous individuals will be mosaics rather than chimeras. While we may expect chimeras to be rare, evidence is accumulating indicating that genetic mosaics are probably the rule while genetic homogeneity, one of the conventional hallmarks of individuality, is the exception (Santelices, 1999; Rinkevich, 2000; Loxdale & Lushai, 2003). High degrees of IGH, i.e. many different genetic lineages within an individual, have been detected in a number of different types of organisms. For example, Thomson *et al.* (1991) sampled leaves from strangler fig trees (2–11 per tree) from several branches in 14 individuals of six species of figs. Thirteen of the individual trees showed multiple genotypes (2–8 genotypes), including the trees where only two leaves had been sampled. All in all the 14 trees represented at least 45 genetic individuals.

6. 3. 1. The genetic mosaicism hypothesis

The genetic mosaicism hypothesis (GMH) was first proposed to explain patterns of coevolution between long-lived plants and their short-lived, and rapidly evolving, herbivores and pests (Whitman & Slobodchikoff, 1981; Gill, 1986). According to the GMH, long-lived arborescent plants accumulate somatic mutations and become genetic mosaics as they grow. The GMH predicted that selection can act on this new genetic material during the somatic stage of ontogeny (Gill *et al.*, 1995). Despite the name, the Genetic Mosaicism Hypothesis, the hypothesis does not require the IGH to be of a mosaic type. It is equally applicable to chimeric heterogeneity and perhaps a more suitable name would be the Genetic Heterogeneity Hypothesis, GHH (Pineda-Krch and Lehtila, 2004). Under the GMH the phenotypic variability of the higher-level unit increases conferring a greater phenotypic repertoire when dealing with environmental contingencies such as herbivory, pests, and micro-evolutionary adaptations (Silander, 1985; Rinkevich & Shapira, 1999).

Herbivores, for example, do not cope well with varying food quality (Suomela & Nilson, 1994; Karban *et al.*, 1997). In long-lived modular organisms, variable module quality entails both a short-term and long-term edge in the interspecific arms race with pests and herbivores. Genetic mosaicism may be one way of producing such variation in quality (Whitham & Slobodchikoff, 1981; Gill & Halversson, 1984). Edwards *et al.* (1990) observed that *Eucalyptus melliodora* trees, which normally suffer from high levels of herbivory by Christmas beetle, *Anoplognathus montanus*, sometimes carry uninfested branches. It turned out that the content of volatile oils between damaged and undamaged leaves differed, which was possibly caused by newly arisen mutations in meristematic tissue. In its simplest form, novel beneficial variants are selected for, as in the case of *E. melliodora*, with the potential of replacing the original variant. In an alternative scenario, however, the resistant leaves may protect vulnerable parts of the plant and thus benefit the whole individual. The GMH is not limited to interactions with pests, conceptually it is equally applicable to any benefit to the individual IGH may have. In chimeric strangler figs (*Ficus* spp.), e.g. IGH may result in flowering asynchrony among genetically distinct branches that have originated by fusion of separate trees (Thomson *et al.*, 1991). Although the benefits of this asynchrony have not been investigated, one potential benefit could be that the figs, which are spaced far apart and depend on wasps for their pollination, could more easily attract and maintain a large enough wasp population for pollination if they flower continuously. Synchronous flowering, on the other hand, may also be beneficial as outcrossing could take place within the crown of a single tree.

6. 3. 2. Plant chimeras and genetic heterogeneity

Chimerism originates from allogenic fusion or grafting whereas mosaicism originates by intrinsic genetic variations caused, among other processes, by somatic mutations. As such, chimerism is much

rarer and involves a much larger genetic change than mosaicism (Santelices, 2004). The cells in the shoot apical meristem of most higher plants are arranged into clonally distinct layers of cells (L1, L2, L3 etc.). (Satina *et al.*, 1940; Satina and Blakeslee, 1941; Tilney-Bassett, 1986). In the meristems of plant chimeras cells from different genotypes coexist. The advancement of protoplast techniques to produce chimeras has rekindled interest in periclinal chimeras from graft unions (Tilney-Bassett 1986). One well-studied periclinal chimera *+Laburnocytisus adamii* C.Schneid., arose in 1825 as an adventitious shoot from the graft union between *Chamaecytisus purpureus* Link, and *Laburnum anagywides* Medik. It was composed of an L1 histogenic layer from *Chamaecytisus* over two histogenic layers (L2, L3) of *Laburnum.* Winkler (1907) tested grafts between tomato and black nightshade using each species as stock and scion (Tilney-Bassett 1986). New plants that arose as adventitious buds on graft unions were composed of genetically distinct tissues and were called chimeras (Tilney-Bassett 1986). Chimeras have been produced from interspecific grafts within *Citrus* (Iwamasa *et al.*, 1977); *Nicotiana* (Marcotrigiano, 1985); *Lycopersicon* (Goffreda *et al.*, 1990); *Solanum* (Taylor & Henshaw 1990); and *Brassica* (Noguchi *et al.*, 1992).

Methods to distinguish somatic hybrids and chimeras from parental types include morphological characteristics, chromosome counts, isozyme patterns, and DNA markers such as restriction patterns of nuclear DNA coding for ribosomal RNA (Tilney-Bassett 1986; Morikawa & Yamada 1992). Flow cytometry also offers a rapid and sensitive means of identifying the parentage of hybrids (Fahleson *et al.*, 1988; Chaput *et al.*, 1990). Hansen *et al.* (2004) used RAPD markers to distinguish donor DNA in a *Populus* chimera. Hocquigny *et al.* (2004) used microsatellite loci to demonstrate the prevalence of periclinal chimeras which contributed to polymorphism between clones of *Vitis vinifera* 'Pinot'.

It is, therefore, desirable to be able to estimate genetic fidelity within vegetatively propagated plants. A variety of methods for the estimation of relatedness or similarity are associated with molecular markers. The most common of these and those used in this study to determine the similarity (or difference) between different samples of the one clone are described below.

6. 4. Data analysis

6. 4. 1. Distance and clustering methods

Estimation of genetic relatedness from microsatellite markers has traditionally been based on the utilisation of algorithms that produce "distance" measures. Distance methods are based on predefined formulae, which estimate the difference between individual samples within a matrix (overall distance) and convert the initial matrix into a "pairwise distance matrix". A common algorithm for creating a

pairwise distance matrix is that of Nei and Li (1979) which measures overall similarity between samples and computes evolutionary distances for all pairs of samples using the formula:

$$\frac{2a}{2a+2b+2c}$$

(a= the number of markers in common, b=the number of markers in one individual and c= the number of markers in the other individual, i.e. making a matching coefficient).

The production of tree diagrams from resulting distance values between samples involves the use of clustering algorithms. Many clustering methods construct a tree by linking the least distant (more similar) pairs of samples, followed by successively more distant taxa. These trees are based solely on the relative numbers of similarities and differences between a set of characters.

Two commonly employed tree building methods are Unweighted Pair Group Method using Arithmetic Averages (UPGMA; Sneath & Sokal, 1973) and Neighbour Joining (NJ; Saitou & Nei, 1987). These methods ultimately produce a single tree, which compared to parsimony methods for example, which involve generation and comparison of a large number of trees, UPGMA and NJ are computationally and temporally rapid for small numbers of samples. Once characters within a matrix are converted to distances, valuable phylogenetic information is lost by not allowing the specific character(s) that contribute to a branch length to be identified. By producing single trees these methods are limited by not allowing competing hypotheses for tree production to be tested (Page and Holmes, 1998). UPGMA is further limited by assuming the rates of change are equal between all samples on a tree (i.e. characters are ultrametric). NJ has a distinct advantage over UPGMA, as it does not assume that all characters are ultrametric. This method involves the modification of the distance matrix to adjust for the differences in the rate of evolution of each sample. The least distant pairs of nodes are linked and their common ancestral node is added to the tree while their terminal nodes are pruned from the tree. This continues until two nodes remain (Saitou and Nei, 1987).

6. 4. 2. Estimation of genetic diversity within the population sample of selected genotypes.

A variety of methods for the estimation of genetic diversity within a population are associated with molecular markers. The most common of these and those used in this study are described below.

The genetic diversity within a population can be described using the following descriptive statistics: the number of alleles produced at each locus and over all loci; observed heterozygosity and expected heterozygosity. Gene diversity is similar to the expected heterozygosity but estimates gene diversity

per locus and sample using an unbiased estimator that allows comparison between different sample sizes (Nei, 1987). Allelic richness, is a measure of the number of alleles independent of sample size, allowing comparison of this quantity between different sample sizes. The observed number of alleles in a sample is highly dependant on sample size. To bypass this problem, El Mousadik & Petit (1996) suggested adaptation of the rarefaction index of Hurlbert (1971) to population genetics.

F-statistics are a set of tools devised by Wright (1921, 1969) to partition heterozygote deficiency into a within and an among population component. F-statistics in population genetics have nothing in common with those used to evaluate differences in variances. In this case, the F stands for fixation index, fixation being increased homozygosity resulting from inbreeding. Population subdivision results in the loss of genetic variation (measured by heterozygosity) within subpopulations due to their being small populations and their being genetic drift acting within each one of them. This would result in decreased heterozygosity relative to that expected heterozygosity under random mating as if the whole population was a single breeding unit. F-statistics are widely used by population biologists to assess levels of structuring in samples of natural populations. F_{is} (the inbreeding coefficient) measures the heterozygote deficit within populations, F_{st} among populations (a measure of the Wahlund effect), and F_{it} the global deficit of heterozygotes.

6. 5. Materials and Methods

6. 5. 1. Collection of plant material

(1) *Estimation of genetic fidelity within clones*

For the analysis of clonal fidelity of plants propagated by grafting and micropropagation techniques, samples were collected from various sources as grafted plants or micropropagated plants (table 6. 2).

- Grafted plants

Superior ash trees were phenotypically selected from the natural Irish population by Coillte. Four scions collected from each selected tree were grafted onto four ash seedling rootstocks. The resultant grafted plants were conserved by planting in the field at Kilmacurra, Co. Wicklow and each genotype designated a unique identity number from 1 to 100 (for a description of these plants refer to chapter 3 section 3. 7. A. (2)). Subsequently, scions from these field-grafts were collected and grafted to seedlings at Teagasc, Kinsealy where they were maintained in the nursery as potted plants in the glasshouse or established in the field. In October 2002, samples from grafted plants of selected genotypes were taken from three different sources including, from the collection of grafted plants grown in the field at Kilmacurra, Co. Wicklow and from field and glasshouse plants in Teagasc,

Kinsealy. From each tree sampled, approximately 10 – 12 leaflets of that years' growth were selected. Care was taken to select leaves from shoots believed to have originated from the original scion wood and not from any shoots produced by the rootstock. It was ensured that each leaflet was relatively disease free (to reduce contamination from DNA of different organisms) before storing them in labeled bags of silica gel (Chase and Hills, 1991). The bags of silica gel ensured rapid desiccation of the leaf material and preservation of the required total DNA until its extraction in the laboratory. One sample from each grafted tree was extracted and the DNA analysed.

- Micropropagated plants

Samples from micropropagated plants were taken from two sources, weaned plants and shoots still in the micropropagation cycle *in vitro*. In October 2002, leaf samples were taken from micropropagated plants of genotype 5, 47, 49, 70 and 72, which were weaned to the glasshouse in the previous year, the leaf samples were stored in silica gel until DNA extraction.

For the *in vitro* material in the micropropagation cycle, three shoots were randomly selected from each genotype 5, 47, 49, 70 and 72. A typical shoot consisted of a stem and numerous leaves (any roots or callus were excluded) the DNA extraction was preformed on fresh material (described in section 6. 5. 2 of this chapter). Genotype 5, 47, 49 and 72 were *in vitro* for approximately 16 months at the time of DNA extraction. All four genotypes had been initiated from previously micropropagated plants weaned to the glasshouse the previous year (also used for DNA sampling). Genotype 70 was initiated in May 1998 from field material collected from grafted plants at Kilmacurra, Co. Wicklow. All cultures were initiated *in vitro* according to the method described for mature trees in chapter 4 section 4. 6. C.

- Labelling of samples

Samples from each location were labelled according to the source code in table 6. 1, the genotype number and the sample letter A to D depending on the number of samples collected from that genotype and source. For example, four grafted plants of genotype 18 were sampled in Kilmacurra, Co. Wicklow and are labelled as K18A, K18B, K18C and K18D.

Table 6. 1. Source codes used to label DNA samples of selected genotypes of *F. excelsior* collected from different locations and used to study genotypic fidelity during vegetative propagation.

Location	*Sample code*
Kilmacurra, Co. Wicklow (Grafted plants)	K
Teagasc Kinsealy Research Centre: Field (Grafted plants)	F
Teagasc Kinsealy Research Centre: Glasshouse (Grafted plants)	G
Teagasc Kinsealy Research Centre: Glasshouse (Micropropagated: weaned)	M
Teagasc Kinsealy Research Centre: Growth room (Micropropagated: *in vitro*)	C

Table 6. 2. The number of plants sampled for nuclear microsatellite DNA analysis of clonal fidelity in *F. excelsior.*

	Grafted plants			Micropropagated plants	
Source/ Genotype	Kilmacurra (K)	Kinsealy field (F)	Kinsealy glasshouse (G)	Weaned (M)	*In vitro* (C)
F5*	-	-	-	4	3
8	4	3	2	-	-
18	4	-	-	-	-
22	4	-	3	-	-
29	3	-	3	-	-
44	3	-	-	-	-
47	4	3	-	3	3
49	4	3	-	3	3
56	4	2	3	-	-
69	3	3	-	-	-
70	4	3	-	4	3
72	4	3	3	4	3
73	4	-	3	-	-

* Material from genotype F5 was not available as grafted plants. The cultures of F5 were established from buds collected directly from the mother tree.

(2) Estimation of genetic diversity within the selected population

Samples for the analysis of genetic diversity within the selected population were collected from grafted plants growing in 3 litre rose pots in the glasshouse at Teagasc, Kinsealy. Grafted plants were available for 90 different selected genotypes and from these, 50 genotypes were randomly selected for

DNA analysis. The location of the original selected trees is shown in figure 6. 2. In some cases more than one selected genotype was sampled from any given location. DNA was extracted and analysed from the leaves of forced cuttings. Three – four stem cuttings containing the dormant leaf buds were taken, from each grafted genotype in February 2004, labelled carefully and given heat treatment to break bud dormancy (described in chapter 3 section 3. 7. D. (3)). When the new leaves emerged (approximately two-three weeks after heat treatment) they were collected and the DNA extraction was performed on the fresh leaf material.

6. 5. 2. DNA Extraction

The total genomic DNA was extracted from the leaves (and stems of the *in vitro* material), this procedure involves treatments that disrupt the plant cell wall and cell membranes to release the cellular constituents into an extraction buffer that contains compounds to protect the DNA from the activity of endogenous nucleases. The conditions of the extraction buffer are critical for successful DNA extraction. The pH of the buffer must be optimised to avoid the optima for degradative enzymes such as DNases and lipoxygenase, which have their optima at pH 5-7 (Hodkinson, personal communication).

The extraction procedures followed the Hot CTAB method of Gawal and Jarret (1991). The detergent CTAB solubilises the plant membranes and forms complexes with the DNA aiding extraction from the plant cells. The various other components of the extraction buffer listed also help protect the extracted DNA from degradation. 10ml of 2× CTAB buffer (100mM Tris-HCl; 1.4M NaCl; 20mM EDTA, 2% CTAB) were added to a 30ml capped centrifuge tube. This was preheated along with a mortar and pestle at 65°C in an oven.

Approximately 200-300mg of silica dried material or 300-600mg of fresh material was used to extract total DNA. The plant material was weighed out and cut into small pieces then placed in a preheated mortar and ground using a small amount of the extraction buffer. The remaining buffer was added slowly and the material further ground to form a suspended slurry. This was carefully poured back into the capped 30ml centrifuge tube and incubated at 65°C for 10 minutes, with occasional shaking.

10ml of chloroform: isoamyl alcohol (24:1 v/v) was then added, this removes constituents solvable in the organic phase such as proteins and lipids in the mixture; it also separates the aqueous (DNA containing) solution from the organic solution. The tubes were placed horizontally on an agitating table for approximately 30 minutes. The tubes were then centrifuged at 1780 rcf for 10 minutes and the upper aqueous (DNA containing) layer was removed and transferred to a fresh, labelled 30ml tube

using a transfer pipette. An equal volume of cold isopropanol (-20°C) was added and the tubes inverted to initially precipitate the DNA. The samples were then placed in a –20°C freezer overnight to further precipitate the DNA.

The next stage involved the purification of the extracted DNA, this employed steps to remove the DNA from the isopropanol and wash it in 70% ethanol. This removes impurities from the precipitate and allows the DNA to be transferred to a storage buffer suitable for freezing and for use in the Polymerase Chain Reaction. After removal from the freezer the samples were centrifuged at 440 rcf for 10 minutes to pellet the DNA. The supernatant was poured off and 3ml of 70% ethanol added. The samples were recentrifuged at 440 rcf for 5 minutes to pellet the DNA once more. The supernatant was poured off and the tubes placed upside down for approximately 5 minutes to let the excess wash buffer drain away. The tubes were then placed upwards in a fume cupboard for approximately 20 minutes to ensure all traces of ethanol were removed. The pellet was then resuspended in 0.5ml of Tris EDTA (TE) buffer (pH 8) (10mM Tris HCl; 1mM EDTA) and transferred to a labelled 1.5 ml microcentrifuge tube using a transfer pipette. The tubes were then stored in a freezer at –20°C until further use.

6. 5. 3. Determination of the fragment size of extracted DNA

It is necessary to estimate the size of DNA fragments (in base pairs) obtained from the extraction process as DNA of high molecular weight is required for the PCR reaction. The fragments of DNA are usually sized by comparing the distance they move during electrophoresis with a standard 1 Kb ladder containing sections of DNA of known molecular weight, run on the same gel.

8 μl of the suspended DNA was loaded with 2μl of the loading dye (blue/orange 6x, Promega, California, USA) into wells on a 1.2% agarose gel (1.2g agarose powder; 100ml 1% TBE Buffer; 2μl ethidium bromide) and covered with 1% Tris: Boric acid: EDTA (TBE) buffer (0.445M Tris-HCl, 0.445M Boric acid, 0.055M disodium EDTA, pH 8.3). The loading dye allows the DNA sample to be visualised and also enables the sample to sink into the wells on the gel. The gel was run at 110 volts for 30 minutes, after which time it was removed and viewed under a UV light source. Each sample was checked to ensure that it contained high molecular weight DNA by subjective comparison with the 1Kb ladder. Unfortunately determination of the molecular weight of the extracted DNA was not possible as the samples were constricted from moving through the gel and as a result most of the fluorescence was in the wells of the gel. This was suspected to be cause by polysaccharides in the DNA extraction that prevent movement through the agarose gel. Spin column purification was therefore required.

6. 5. 4. Spin Column Purification of the extracted DNA

A Jet Quick™ PCR Purification System (Genomed, GmBH, Germany) was employed to further purify the samples. This procedure was carried out to remove any impurities from the extract, which may interfere with the PCR. Before beginning, an aliquot of TE Buffer was preheated to 65°C. It was also verified that ethanol had been added to the wash buffer. The sample was prepared by adding 100µl of the original total DNA sample to 400µl of Binding Solution (concentrated guanidine hydrochloride, EDTA, Tris-HCl, isopropanol) in a labelled 1.5ml microcentrifuge tube and gently mixing. A spin cartridge was then placed into a 2ml wash tube. The sample from the 1.5ml microcentrifuge tube was then loaded into the spin cartridge. The mixture was centrifuged at 12,000 rcf for 1 minute in a microcentrifuge. The flow through was discarded and the spin cartridge placed back into the 2ml wash tube. 700µl of wash buffer (containing ethanol) was added to the spin cartridge. This was centrifuged at 12,000 rcf for 1 minute. The flow through was discarded and the mixture recentrifuged at 12,000 rcf for 1 minute to remove all residual wash buffer. The spin cartridge was then placed into a fresh, labelled 1.5ml recovery tube and 50µl of the warm TE buffer added directly to the centre of the spin cartridge. This was incubated at room temperature for 1 minute, then centrifuged at 12,000 rcf for 2 minutes. The spin cartridge was removed from the recovery tube and discarded. The recovery tubes containing the purified DNA were then stored in the freezer at –20°C until further use. After Spin Column Purification each sample was checked on a 1.2% agarose gel (described in section 6. 5. 1) to check DNA quality.

6. 5. 5. Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in a Gene Amp® PCR system 9700. The total PCR volume per tube was 25µl. A 0.5µl (± 100ng DNA) aliquot of the extracted DNA samples was placed in a labelled 0.2ml Dome cap micro-centrifuge tube (see table 6. 3). The remaining volume was prepared as a general master mix and added to the aliquot of DNA just before beginning the reaction. This ensures efficiency and the uniformity of added reagents to each tube. Along with the aliquot of DNA, each tube contained 11.375µl sterile ultrapure water, 5µl of 10x Reaction Buffer (without $MgCl_2$) (500mM KCl; Tris-HCl (pH9); 1%Triton®X-100) (Promega), 1µl (each at 10mM) of dNTPs (Promega), 2.5µl (each at 4%) of forward and reverse primer (MWG-Biotech, North Carolina, U.S.A) 2.5µl (25mM stock) of magnesium chloride and 0.125µl (5u/ul) of *Taq* DNA polymerase (Promega).

Table 6. 3. Microsatellite primer pairs used in the PCR, including locus name, repeat motif (from original publication), primer sequences and specific annealing temperature (T_a).

Locus	Repeat motif	Primer sequence	T_a (^{O}C)
M2-30*	$(TG)_{15}(AG)_{23}$	F: CGCACGTTCTTTCTATTG	56
		R: GACCGGCTGACTATTTTCTC	
FEMSATL 4**	$(CA)_2(AG)_{24}$	F: TTCATGCTTCTCCGTGTCTC	52
		R: GCTGTTTCAGGCGTAATGTG	
FEMSATL 11**	$(GA)_{20}(TA)_4$	F: GATAGCACTATGAACACAGC	52
		R: TAGTTCTACTACTTCAAGAA	
FEMSATL 16**	$(CA)_3CG(CA)_{10}$	F: TTAACAGTTAACTCCCTTC	52
		R: CAACATACAGCTACTAATCA	
FEMSATL 19**	$(CA)_6CGGC(CA)_{13}$	F: CTGTTCAATCAAAGATCTCA	52
		R: TGCTCGCATATGTGCAGATA	

* Brachet *et al.* 1999, **Lefort *et al.* 1999

The appropriate volume of the master mix was added to the template DNA giving the required reaction volume of 25µl. The sealed tubes were then mixed on a vortex, placed in a micro-centrifuge and pulsed for approximately 7 seconds in order to spin down the reagents. The tubes were then loaded into the thermal cycler, which had been programmed with primer specific cycling parameters (table 6. 4).

Table 6. 4. The cycling parameters (in sequential order) for the amplification of DNA by the microsatellite primers.

	Temperature (^{O}C)	Time
Premelt	95	4 minutes
Denature	94	30 seconds
Anneal	See table 3.	45 seconds
Extension	72	1 minute
Final Extension	72	5 minutes
Soak	4	∝
Number of Cycles	-	36

6. 5. 6. Visualisation of the PCR products

6.5μl of PCR product was mixed with 2μl of the loading dye and loaded onto a 1.2% agarose gel covered with 1% TBE Buffer. The gel was run at 110V for 30 minutes. Following electrophoresis, the ethidium bromide stained gel was placed over a UV light source. Each sample could then be checked for amplification products.

6. 5. 7. Preparation of samples for analysis on the ABI PRISM™ 310 Genetic Analyser.

Microsatellite allele sizing was carried out on an ABI PRISM™ 310 Genetic Analyser (Applied Biosystems, California, U.S.A). Each primer pair used in the PCR has its reverse primer labelled with a fluorescent dye so when the samples are run on the genetic analyser three samples of different colour can be placed in the same tube and run simultaneously (multiplexing). The primers were labelled as follows (table 6. 5):

Table 6. 5. The fluorescent label colour and name of the microsatellite primers used to analyse the breeding population of *F. excelsior.*

Primer	*Label Colour*	*Label Name*
M2-30	Green	JOE
FEMSATL 4	Blue	FAM
FEMSATL 11	Yellow	TAMRA
FEMSATL 16	Green	JOE
FEMSATL 19	Green	JOE

For analysis by the genetic analyser it is only necessary to have very small volumes of PCR product. After PCR amplification and confirmation of the presence of amplification products by gel electrophoresis it was therefore necessary to dilute the samples. Samples were diluted with ultrapure water as follows (table 6. 6):

Table 6. 6. The dilution ratio and reaction volume of the fluorescent labelled microsatellite PCR products used for analysis on the genetic analyser.

Label Name	*Dilution ratio (Sample: SDDW)*	*Vol. added to tubes*
FAM	1:30	0.6µl
JOE	1:15	0.8µl
TAMRA	1:60	1.3µl

The amplification products were diluted and combined in 0.5ml tubes, using the quantities outlined in table 6. 6, with 0.25µl Genescan™ 500 ROX™ size standard (30-500bp) (Applied Biosystems) and 24µl of Formamide, which is used to denature the DNA samples. The samples were then denatured on a heating block for 10 minutes at 95°C, transferred to an ice tray and centrifuged. The samples were then loaded onto the ABI PRISM® 310 Genetic Analyzer. The module used was GS STR POP 4 (1ml) A. Genescan® analysis software version 3.1 was used to read and size each sample using comparisons with the ROX size standard. Genotyper® Software Version 3.7. was used to visualise and score the microsatellite profile for each sample.

6. 5. 8. Data analysis

(1) Estimation of genetic fidelity within clones

DNA fragments from each locus were scored as presence/ absence characters and were analysed with neighbour joining (NJ) using Nei & Li distances (average difference (band sharing) between individuals) (Nei & Li, 1985), mean character difference and total character difference (total number of character (allelic) differences between individuals) with the phylogenetic program PAUP 4.0bv10 (Swofford, 2003).

(2) Estimation of genetic diversity within the selected population

For analytical purposes, the group of selected ash trees were considered as a single "population", even though their origin was from different locations in Ireland (figure 6. 2). The observed and expected heterozygosities were computed using the software MSAnalyser (version 3.15, Dieringer and Schlotterer, 2003). The number of alleles and allelic richness were computed using the software FSTAT (version 2.9.3.2., Goudet, 1995), gene diversity was estimated according to Nei (1987), and inbreeding coefficient F_{is} (Wright, 1969) using the FSTAT software. The genetic diversity indices of the selected population were compared with those of three natural Irish populations and material from 14 natural provenances in Ireland (Harbourne, 2004).

6. 6. Results

6. 6. 1. Genetic fidelity within genotypes

The number of samples successfully amplified from each genotype are listed in table 6. 7. Two DNA samples failed to amplify using the five microsatellite primers M2-30, FEMSATL 4, 11, 16 and 19, and one sample only amplified using FEMSATL 4 and 19, these were all discarded from the data set. Three samples produced amplification products for four primers and all the other samples produced amplification products for all five primers.

Table 6. 7. The number samples successfully amplified for nuclear microsatellite DNA analysis of clonal fidelity in *F. excelsior*.

	Grafted plants			*Micropropagated plants*	
Source/ Genotype	Kilmacurra (K)	Kinsealy field (F)	Kinsealy glasshouse (G)	Weaned (M)	*In vitro* (C)
F5 (05)	-	-	-	4	3
08	4	3	2	-	-
18	4	-	-	-	-
22	4	-	3	-	-
29	3	-	3	-	-
44	3	-	-	-	-
47	4	3	-	3	3
49	4	3	-	2	3
56	4	2	3	-	-
69	3	3	-	-	-
70	4	3	-	4	3
72	4	1	3	4	3
73	4	-	3	-	-

Each of the five microsatellite primers tested successfully detected polymorphisms within the clones of *F. excelsior*. Tables showing the actual allele sizes scored for each locus and sample are located in appendix 6. Table 6. 8 contains a summary of the number of alleles and the allelic size range per locus. The NJ tree produced from the Nei-li distances (figure 6. 1.) illustrates the grouping of samples from each genotype depending on their similarity to each other.

Table 6. 8. The number of alleles and the allelic size range per locus for five microsatellite loci used to test the genetic fidelity within the vegetative propagules of 13 ash trees.

Locus	*Number of alleles*	*Allele size range*
M2-30	36	173-247
FEMSATL 4	30	160-256
FEMSATL 11	17	184-228
FEMSATL 16	11	161-212
FEMSATL 19	28	143-210
Total	122	n/a*

Genotype F5

Material from grafted plants of genotype F5 were not available for testing. The results from weaned and micropropagated plants of genotype F5 (labelled 05 in this study) are presented in table 6. 9 and on the NJ tree (figure 6.1). Polymorphisms were detected within the weaned plants at the loci FEMSATL 4, 19 and M2-30 (appendix 6 table 4). None of the weaned plants were identical to one another and total character differences of between 2 and 9 were recorded. Two out of the three *in vitro* shoots were identical (total character difference of 0) and one had two polymorphic bands at the M2-30 locus. In total, five out of the seven micropropagated plants (weaned plants and *in vitro* shoots) tested showed genetic variation. However, all plants of genotype F5 are grouped together on the NJ tree.

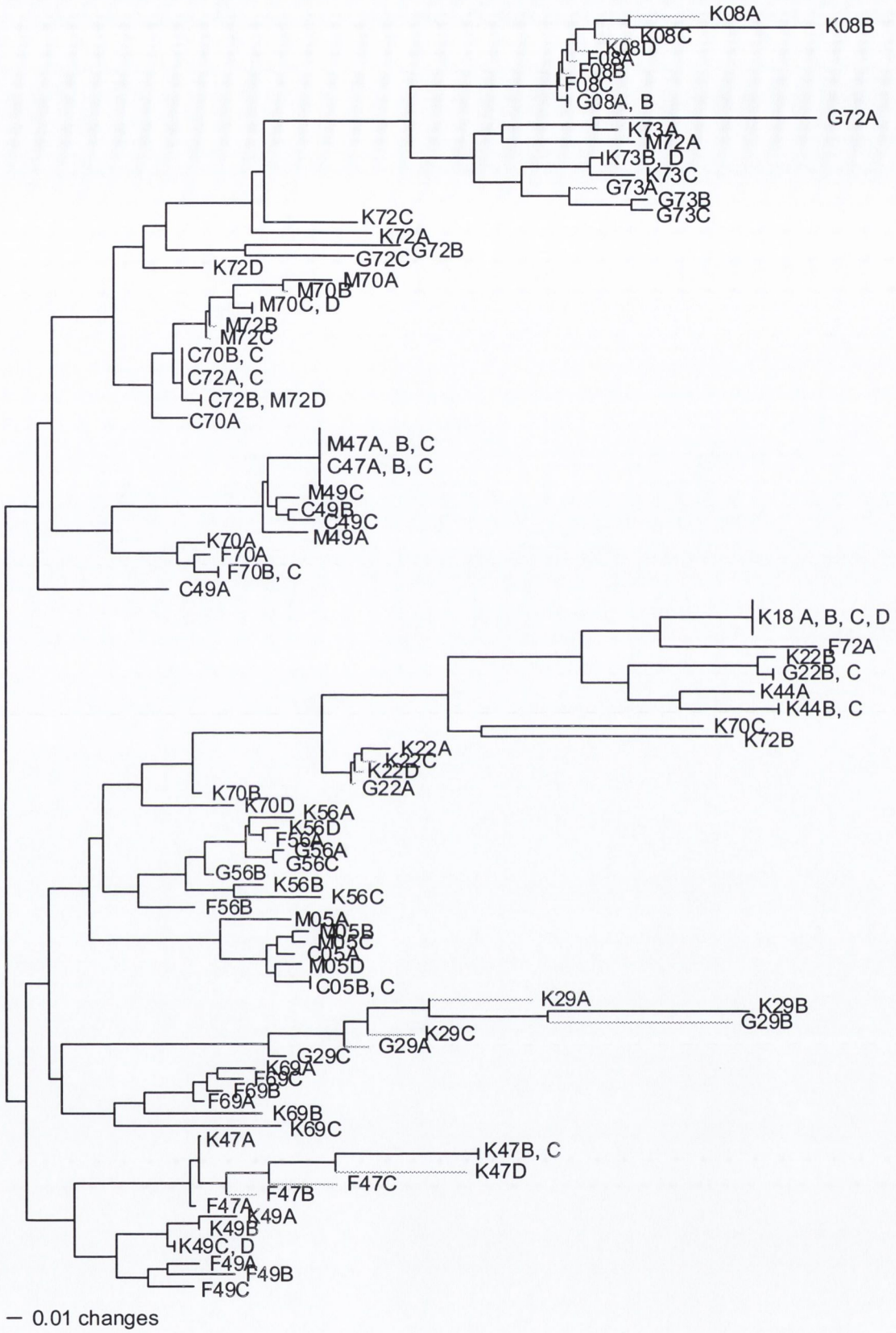


Figure 6. 1. Neighbour joining (NJ) tree of microsatellite data. Branch length is proportional to genetic distance according to Nei-Li (mid point rooted).

Table 6. 9. The total and mean character differences detected across all loci between samples of genotype F5 (labeled as 05 in this study).

	*M05A	M05B	M05C	M05D	C05A	C05B	C05C
M05A	-**	0.052	0.037	0.067	0.052	0.067	0.067
M05B	7	-	0.015	0.045	0.029	0.045	0.045
M05C	5	2	-	0.029	0.015	0.029	0.029
M05D	9	6	4	-	0.029	0.029	0.029
C05A	7	4	2	4	-	0.029	0.029
C05B	9	6	4	4	4	-	**0.000**
C05C	9	6	4	4	4	**0**	-

*M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant **Below diagonal: Total character differences; above diagonal: Mean character differences.

Genotype 8

The results for genotype 8 are presented in table 6. 10 and on the NJ tree (figure 6. 1). Polymorphisms were detected between plants at the loci FEMSATL 16 and 19 (appendix 6 table 5). Three out of the four grafted plants at Kilmacurra were identical to each other, the odd one (K08B) had two polymorphic bands at FEMSATL 19. The field plants in Kinsealy were identical to each other and were identical to three of the four plants at Kilmacurra (K08A, C, D). The plants in the glasshouse in Kinsealy (G08A, B) were identical to each other but differed from the Kilmacurra plants by 1-7 characters and from the Kinsealy field plants by 1 character. The polymorphism between the identical plants at Kilmacurra (K08A, C, D) and Kinsealy field (F08A-C) and the Kinsealy glasshouse plants was identified as an extra band recorded at the FEMSATL 16 locus. Five out of the nine grafted plants of genotype 8 tested were identical. All plants of genotype 8 are grouped together on the NJ tree.

Table 6. 10. The total and mean character differences detected across all loci between samples of genotype 8.

	*K08A	K08B	K08C	K08D	F08A	F08B	F08C	G08A	G08B
K08A	-**	0.045	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	0.007	0.007
K08B	6	-	0.045	0.045	0.045	0.045	0.045	0.052	0.052
K08C	**0**	6	-	**0.000**	**0.000**	**0.000**	**0.000**	0.007	0.007
K08D	**0**	6	**0**	-	**0.000**	**0.000**	**0.000**	0.007	0.007
F08A	**0**	6	**0**	**0**	-	**0.000**	**0.000**	0.007	0.007
F08B	**0**	6	**0**	**0**	**0**	-	**0.000**	0.007	0.007
F08C	**0**	6	**0**	**0**	**0**	**0**	-	0.007	0.007
G08A	1	7	1	1	1	1	1	-	**0.000**
G08B	1	7	1	1	1	1	1	**0**	-

*K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); G=Grafted (Kinsealy glasshouse) **Below diagonal: Total character differences; above diagonal: Mean character differences

Genotype 18

The results for genotype 18 are illustrated on the NJ tree (figure 6. 1). In genotype 18 all the plants tested from Kilmacurra were genetically identical to each other (total character difference of 0).

Genotype 22

The results for genotype 22 are presented in table 6. 11 and on the NJ tree (figure 6.1). Polymorphisms were detected between plants at the loci FEMSATL 11 and 16 (appendix 6 table 7). Two out of the four grafted plants at Kilmacurra were identical to each other (K22C and D), compared to these plants, K22A had a missing band at the FEMSATL 11 locus and K22B had a homozygous polymorphic band at the FEMSATL 16 locus. The Kinsealy glasshouse plant G22A was identical to the two Kilmacurra plants K22C and D, and compared to these plants both G22B and C were missing one band at the FEMSATL 16 locus. Three out of the seven grafted plants of genotype 22 were identical. In the NJ tree plants of genotype 22 are divided into two separate groups K22A, C, D and G22A are grouped together whereas, K22B and G22B and C are in a separate group (however all the plants group at a lower level in the tree).

Table 6. 11. The total and mean character differences detected across all loci between samples of genotype 22.

	*K22A	*K22B*	*K22C*	*K22D*	*G22A*	*G22B*	*G22C*
K22A	-**	0.031	0.007	0.007	0.007	0.015	0.015
K22B	4	-	0.022	0.022	0.022	0.015	0.015
K22C	1	3	-	**0.000**	**0.000**	0.007	0.007
K22D	1	3	**0**	-	**0.000**	0.007	0.007
G22A	1	3	**0**	**0**	-	0.007	0.007
G22B	2	2	1	1	1	-	**0.000**
G22C	2	2	1	1	1	**0**	-

*K= Grafted (Kilmacurra); G=Grafted (Kinsealy glasshouse) **Below diagonal: Total character differences; above diagonal: Mean character differences.

Genotype 29

The results for genotype 29 are shown in table 6. 12 and on the NJ tree (figure 6.1). Polymorphisms were detected between plants at the loci FEMSATL 4, 16 and M2-30 (appendix 6 table 8). For K29B, FEMSATL 16 failed to produce any amplification products, but all other loci had identical bands to K29A. K29A and C differed by and extra band at the FEMSATL 16 locus in K29A and an extra band at the FEMSATL 4 locus in K29C. G29A was identical to K29C and was the only plant in the glasshouse at Kinsealy to have the extra band at FEMSATL 4. G29C differed from all the plants tested by having two polymorphic bands at the M2-30 locus. Only two out of the six grafted plants of genotype 29 were identical. All plants of genotype 29 grouped together in the NJ tree.

Table 6. 12. The total and mean character differences detected across all loci between samples of genotype 29.

	*K29A	K29B	K29C	G29A	G29B	G29C
K29A	-**	0.015	0.015	0.015	0.007	0.037
K29B	2	-	0.015	0.015	0.007	0.037
K29C	2	2	-	**0.000**	0.007	0.037
G29A	2	2	**0**	-	0.007	0.037
G29B	1	1	1	1	-	0.029
G29C	5	5	5	5	4	-

*K= Grafted (Kilmacurra); G=Grafted (Kinsealy glasshouse) **Below diagonal: Total character differences; above diagonal: Mean character differences.

Genotype 44

The results for genotype 44 are shown on the NJ tree (figure 6.1). In genotype 44, two out of the three plants at Kilmacurra were identical (K44B and C), the plant K44A had polymorphisms at four loci (FEMSATL 11, 16 19 and M2-30) resulting in a total character difference of 8. The polymorphisms detected included bands of different length compared to those recorded for K44B and C and extra bands in homozygous loci (appendix 6 table 9). All plants of genotype 44 are grouped together on the NJ tree.

Genotype 47

The results for genotype 47, are shown in table 6. 13 and on the NJ tree (figure 6.1). Polymorphisms within clones were detected at all five loci tested (appendix 6 table 10). Two of the grafted plants at Kilmacurra were identical to each other (K47B and C), K47A differs from K47D by one polymorphic allele at the FEMSATL 4 locus and both these plants differ from K47B and C by having a second allele at the FEMSATL 16 locus. One of the Kinsealy field plants (F47C) is identical to K47D. F47A differs from F47C by one polymorphic allele at the FEMSATL 4 locus and F47B differs from F47A and F47C by being homozygous for one allele at the FEMSATL 4 locus. All the micropropagated plants tested (weaned and *in vitro*) were identical to each other but differed from the grafted plants at all five loci. Of the grafted plants, three out of seven were identical. The grafted plants of genotype 47 are grouped together on the NJ tree, whereas the micropropagated plants are placed together in a separate group some distance away and close to the micropropagated plants of genotype 49.

Genotype 49

The results for genotype 49 are shown in table 6. 14 and on the NJ tree (figure 6.1). Polymorphisms within clones were detected at all five microsatellite loci tested (appendix 6 table 11). Two of the grafted plants at Kilmacurra were identical (K49C and D), K49A and B differed from these plants by the same allele at FEMSATL 4, furthermore, K49B was homozygous at the FEMSATL 16 locus, whereas

all the other plants were heterozygotes at this locus. None of the plants at the field in Kilmacurra were identical to the plants in the field at Kinsealy and none of the Kinsealy field plants were identical to each other. Polymorphisms were detected between the Kinsealy field plants at the FEMSATL 4, 19 and M2-30 loci. None of the micropropagated plants tested were identical to each other or to any of the grafted plants, polymorphisms were detected in four out of the five loci tested, only the homozygous allele at FEMSATL 16 remained constant between the samples. In fact, most of the micropropagated plants of genotype 49 are grouped with the micropropagated plants of genotype 47 in the neighbour joining tree. For genotype 49, only two out of seven grafted plants were identical and no micropropagated plants were identical. However, all the grafted plants of genotype 49 are grouped together in the NJ tree. Only C49A is placed on its own on the NJ tree away from both the micropropagated and grafted plants of genotype 49.

Table 6. 13. The total and mean character differences detected across all loci between samples of genotype 47.

	*K47A	K47B	K47C	K47D	F47A	F47B	F47C	M47A	M47B	M47C	C47A	C47B	C47C
K47A	-**	0.022	0.022	0.015	0.015	0.022	0.015	0.127	0.127	0.127	0.127	0.127	0.127
K47B	3	-	**0.000**	0.007	0.022	0.015	0.007	0.134	0.134	0.134	0.134	0.134	0.134
K47C	3	**0**	-	0.007	0.022	0.015	0.007	0.134	0.134	0.134	0.134	0.134	0.134
K47D	2	1	1	-	0.015	0.007	**0.000**	0.127	0.127	0.127	0.127	0.127	0.127
F47A	2	3	3	2	-	0.022	0.015	0.112	0.112	0.112	0.112	0.112	0.112
F47B	3	2	2	1	3	-	0.007	0.119	0.119	0.119	0.119	0.119	0.119
F47C	2	1	1	**0**	2	1	-	0.127	0.127	0.127	0.127	0.127	0.127
M47A	17	18	18	17	15	16	17	-	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
M47B	17	18	18	17	15	16	17	**0**	-	**0.000**	**0.000**	**0.000**	**0.000**
M47C	17	18	18	17	15	16	17	**0**	**0**	-	**0.000**	**0.000**	**0.000**
C47A	17	18	18	17	15	16	17	**0**	**0**	**0**	-	**0.000**	**0.000**
C47B	17	18	18	17	15	16	17	**0**	**0**	**0**	**0**	-	**0.000**
C47C	17	18	18	17	15	16	17	**0**	**0**	**0**	**0**	**0**	-

*K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant **Below diagonal: Total character differences; above diagonal: Mean character differences.

Table 6. 14. The total and mean character differences detected across all loci between samples of genotype 49.

	*K49A	K49B	K49C	K49D	F49A	F49B	F49C	M49A	M49C	C49A	C49B	C49C
K49A	-**	0.014	0.022	0.022	0.059	0.052	0.067	0.104	0.097	0.052	0.089	1.104
K49B	2	-	0.022	0.022	0.059	0.067	0.067	0.104	0.097	0.052	0.089	0.104
K49C	3	3	-	**0.000**	0.037	0.045	0.045	0.112	0.104	0.045	0.097	0.112
K49D	3	3	**0**	-	0.037	0.045	0.045	0.112	0.104	0.045	0.097	0.112
F49A	8	8	5	5	-	0.037	0.037	0.119	0.112	0.067	0.104	0.112
F49B	7	9	6	6	5	-	0.045	0.097	0.089	0.045	0.082	0.097
F49C	9	9	6	6	5	6	-	0.112	0.104	0.075	0.082	0.097
M49A	14	14	15	15	16	13	15	-	0.037	0.082	0.045	0.029
M49C	13	13	14	14	15	12	14	5	-	0.089	0.022	0.022
C49A	7	7	6	6	9	6	10	11	12	-	0.082	0.097
C49B	12	12	13	13	14	11	11	6	3	11	-	0.015
C49C	14	14	15	15	16	13	13	4	3	13	2	-

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant. ** Below diagonal: Total character differences; above diagonal: Mean character differences.

Genotype 56

The results for genotype 56 are shown in table 6. 15 and on the NJ tree (figure 6.1). Polymorphisms between the plants were detected by four out of the five loci tested (appendix 6 table 12). Only the heterozygous alleles at the FEMSATL 11 remained constant between all the plants tested. None of the grafted plants at Kilmacurra were identical to each other, or to either the plants in the field or glasshouse at Kinsealy. The field plants in Kinsealy were different from each other and from the plants in the glasshouse in Kinsealy which also differed from each other. Between all nine grafted plants tested there were eight alleles recorded at FEMSATL 4, at FEMSATL 16 eight out of the nine plants tested had the same heterozygous alleles, however, one plant was homozygous for one of the alleles. There were six different alleles recorded between all the plants at the FEMSATL 19 locus and the M2-30 locus. However, all the plants tested of genotype 56 are grouped together in the NJ tree.

Table 6. 15. The total and mean character differences detected across all loci between samples of genotype 56.

	*K56A	K56B	K56C	K56D	F56A	F56B	G56A	G56B	G56C
K56A	-**	0.045	0.089	0.037	0.029	0.059	0.045	0.052	0.037
K56B	6	-	0.045	0.022	0.015	0.059	0.045	0.037	0.037
K56C	12	6	-	0.067	0.059	0.074	0.089	0.082	0.082
K56D	5	3	9	-	0.007	0.052	0.037	0.045	0.029
F56A	4	2	8	1	-	0.045	0.029	0.037	0.022
F56B	8	8	10	7	6	-	0.059	0.067	0.052
G56A	6	6	12	5	4	8	-	0.037	0.007
G56B	7	5	11	6	5	9	5	-	0.029
G56C	5	5	11	4	3	7	1	4	-

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); G=Grafted (Kinsealy glasshouse). ** Below diagonal: Total character differences; above diagonal: Mean character differences.

Genotype 69

The results for genotype 69 are shown in table 6. 16 and on the NJ tree (figure 6.1). Polymorphisms between the plants were detected by four out of the five loci tested (appendix 6 table 13). Only the heterozygous alleles at the FEMSATL 16 remained constant between all the plants tested. None of the grafted plants at Kilmacurra were identical to each other, or to the plants in the field at Kinsealy which also differed from each other. Four polymorphic alleles between all plants were recorded from FEMSATL 4, three from FEMSATL 11 and 19 and four from M2-30. However, all the plants in genotype 69 are grouped together in the NJ tree.

Table 6. 16. The total and mean character differences detected across all loci between samples of genotype 69.

	*K69A	K69B	K69C	F69A	F69B	F69C
K69A	-**	0.052	0.075	0.029	0.022	0.022
K69B	7	-	0.067	0.052	0.045	0.059
K69C	10	9	-	0.059	0.052	0.067
F69A	4	7	8	-	0.007	0.022
F69B	3	6	7	1	-	0.015
F69C	3	8	9	3	2	-

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field). ** Below diagonal: Total character differences; above diagonal: Mean character differences.

Genotype 70

The results for genotype 70 are shown in table 6. 17 and on the NJ tree (figure 6.1). Polymorphisms between samples were detected by all five loci tested (appendix 6 table 14). All the grafted plants at Kilmacurra showed considerable variation at each locus, and are not grouped together on the

neighbour joining tree. However, the three plants in the field at Kinsealy were similar to K70A and all four plants are grouped together by the neighbour joining tree. This may indicate that the grafted plants at Kinsealy were derived from scion wood collected from tree K70A at Kilmacurra. Two of the plants in the field at Kinsealy are identical (F70B and C), the third F70A differs from these plants at the M2-30 locus. The weaned micropropagated plants show considerable variation from the all the grafted plants and are grouped separately from these in the NJ tree (figure 6. 1). The micropropagated weaned plants and *in vitro* shoots of genotype 70 are grouped closely with the micropropagated weaned and *in vitro* shoots of genotype 72 in the NJ tree, in fact C70B and C are considered identical to C72A and C.

Table 6. 17. The total and mean character differences detected across all loci between samples of genotype 70.

	*K70 A	K70 B	K70 C	K70 D	F70 A	F70 B	F70 C	M70 A	M70 B	M70 C	M70 D	C70 A	C70 B	C70 C
K70 A	-**	0.082	0.089	0.097	0.022	0.022	0.022	0.059	0.059	0.067	0.067	0.059	0.067	0.067
K70 B	11	-	0.082	0.074	0.089	0.089	0.089	0.069	0.067	0.059	0.059	0.094	0.074	0.074
K70 C	12	10	-	0.052	0.097	0.097	0.097	0.089	0.089	0.097	0.097	0.104	0.097	0.097
K70 D	13	10	7	-	0.104	0.104	0.104	0.097	0.097	0.089	0.098	0.097	0.089	0.089
F70 A	3	12	13	14	-	0.015	0.015	0.067	0.067	0.075	0.075	0.067	0.074	0.074
F70 B	3	12	13	14	2	-	**0.000**	0.067	0.067	0.075	0.075	0.067	0.074	0.074
F70 C	3	12	13	14	2	**0**	-	0.067	0.067	0.075	0.075	0.067	0.074	0.074
M70 A	8	9	12	13	9	9	9	-	0.015	0.037	0.037	0.074	0.052	0.052
M70 B	8	9	12	13	9	9	9	2	-	0.022	0.022	0.059	0.037	0.037
M70 C	9	2	13	12	10	10	10	5	3	-	**0.000**	0.052	0.029	0.029
M70 D	9	2	13	12	10	10	10	5	3	**0**	-	0.052	0.029	0.029
C70 A	8	13	14	13	9	9	9	10	8	7	7	-	0.022	0.022
C70 B	9	10	13	12	10	10	10	7	5	4	4	3	-	**0.00**
C70 C	9	10	13	12	10	10	10	7	5	4	4	3	**0**	-

*K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant. ** Below diagonal: Total character differences; above diagonal: Mean character differences.

Table 6. 18. The total and mean character differences detected across all loci between samples of genotype 72.

	*K72 A	K72 B	K72 C	K72 D	F72 A	G72 A	G72 B	G72 C	M72 A	M72 B	M72 C	M72 D	C72 A	C72 B	C72 C
K72 A	-**	0.082	0.067	0.082	0.126	0.112	0.097	0.089	0.104	0.082	0.082	0.075	0.082	0.075	0.082
K72 B	11	-	0.059	0.104	0.119	0.119	0.104	0.112	0.112	0.104	0.104	0.097	0.104	0.097	0.104
K72 C	9	8	-	0.089	0.134	0.119	0.104	0.097	0.119	0.089	0.089	0.082	0.089	0.082	0.089
K72 D	11	14	12	-	0.134	0.104	0.104	0.082	0.067	0.044	0.044	0.067	0.059	0.067	0.059
F72 A	17	16	18	18	-	0.119	0.119	0.126	0.112	0.119	0.119	0.112	0.119	0.112	0.119
G72 A	15	16	16	14	16	-	0.059	0.067	0.097	0.104	0.104	0.097	0.104	0.097	0.104
G72 B	13	14	14	14	16	8	-	0.082	0.112	0.089	0.089	0.082	0.089	0.082	0.089
G72 C	12	15	13	11	17	9	11	-	0.104	0.082	0.082	0.059	0.067	0.059	0.067
M72 A	14	15	15	9	15	13	15	14	-	0.022	0.022	0.044	0.037	0.044	0.037
M72 B	11	14	12	6	16	14	12	11	3	-	**0.000**	0.022	0.015	0.022	0.015
M72 C	11	14	12	6	16	14	12	11	3	**0**	-	0.022	0.015	0.022	0.015
M72 D	10	13	11	9	15	13	11	8	6	3	3	-	0.007	**0.000**	0.007
C72 A	11	14	12	8	16	14	12	9	5	2	2	1	-	0.007	**0.000**
C72 B	10	13	11	9	15	13	11	8	6	3	3	**0**	1	-	0.007
C72 C	11	14	12	8	16	14	12	9	5	2	2	1	**0**	1	-

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); G=Grafted (Kinsealy glasshouse); M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant. ** Below diagonal: Total character differences; above diagonal: Mean character differences.

Genotype 72

The results for genotype 72 are shown in table 6. 18 and on the NJ tree (figure 6.1). Polymorphisms between samples were detected by all five loci tested. All the grafted plants at Kilmacurra showed considerable variation particularly at the FEMSATL 19 and M2-30 loci where almost every plant had a unique set of alleles. The grafted plants at the field and glasshouse in Kinsealy were not identical to each other or to any of the plants at Kilmacurra. The weaned plant M72A is grouped with plants of genotype 73 on the neighbour joining tree. Two of the weaned micropropagated plants were identical to each other (M72B and C) and one of the *in vitro* shoots (C72B) was identical to the weaned plant M72D. The other two *in vitro* shoots (C72 A and C) were identical to each other and also to C70B and C. All the micropropagated weaned and *in vitro* shoots of genotype 72 (except M72A) are grouped on the same branch of the NJ tree and are intermingled with micropropagated weaned and *in vitro* shoots of genotype 70.

Genotype 73

The results for genotype 73 are shown in table 6. 19 on the NJ tree (figure 6.1). Polymorphisms between the plants were detected at four out of the five loci tested, only the homozygous allele at FEMSATL 16 remained constant between all the plants. Two of the plants (K73B and D) were identical to each other, K73A differed from these plants at four loci where as K73C only differed at the M2-30 loci. None of the plants in the glasshouse at Kinsealy were identical to the Kilmacurra plants or to each other. Variation between the Kinsealy glasshouse plants was only found at the FEMSATL 19 locus. However, all the plants in genotype 73 are grouped together in the NJ tree.

Table 6. 19. The total and mean character differences detected between samples of genotype 73.

	**K73A*	*K73B*	*K73C*	*K73D*	*G73A*	*G73B*	*G73C*
K73A	-**	0.082	0.075	0.082	0.082	0.075	0.075
K73B	11	-	0.022	**0.000**	0.029	0.052	0.052
K73C	10	3	-	0.022	0.052	0.075	0.075
K73D	11	**0**	3	-	0.029	0.052	0.052
G73A	11	4	7	4	-	0.022	0.022
G73B	10	7	10	7	3	-	0.015
G73C	10	7	10	7	3	2	-

* K= Grafted (Kilmacurra); G=Grafted (Kinsealy glasshouse). ** Below diagonal: Total character differences; above diagonal: Mean character differences.

6. 6. 2. Genetic diversity within the selected population

The population of selected genotypes at Kinsealy, Co. Dublin.

A map showing the locations of the selected trees in woods around Ireland is shown in Figure 6. 2. All 50 samples tested in this study produced amplification products with all five microsatellite primers tested. A total of 141 alleles were generated from five microsatellite loci tested (table 6. 20.). All five microsatellite loci scored were highly polymorphic, displaying a high number of alleles (from 11 to 46 alleles per locus) and broad size range of PCR products (table 6. 20.). The observed heterozygosity (H_O) was lower than the expected heterozygosity (H_E) for all loci resulting in positive inbreeding coefficients (F_{is}). The mean inbreeding coefficient (F_{is}) for all loci was 0.32 (F_{is} values ranged from 0.18 to 0.64). The positive values of the inbreeding coefficient indicate a heterozygote deficiency in the population. Hardy-Weinberg equilibrium (HWE) was rejected for all loci ($p<0.01$), all showed a significant deficiency of heterozygotes.

Figure 6. 2. Map of Ireland showing the locations where seed was collected for the provenance trial (red boxes) and the locations of the selected trees used in the genetic diversity study (blue circles).

Table 6. 20. Allelic diversity of five microsatellite loci scored in a sample of selected genotypes from the Irish selected population (Kinsealy) of *Fraxinus excelsior* (sample size (n) =50).

Locus	*No. Alleles***	*Range of sizes (bp)*	H_O	H_E	*Gene diversity*	F_{is}
M2-30	46	175-271	0.70	0.98	0.98	0.28*
FEMSATL 4	32	160-218	0.76	0.95	0.95	0.20*
FEMSATL 11	24	174-232	0.56	0.91	0.91	0.40*
FEMSATL 16	11	161-201	0.24	0.66	0.66	0.64*
FEMSATL 19	28	167-208	0.78	0.93	0.93	0.18*
Total	141	-	0.61	0.88	0.88	0.32*

H_O, observed heterozygosity, H_E, expected heterozygosity and F_{is}, inbreeding coefficient. * F_{is} is significantly different from Hardy-Weinberg equilibrium (HWE), $p<0.01$. ** Equivalent to allelic richness.

Table 6. 21. Allelic diversity of five microsatellite loci scored in a sample of *Fraxinus excelsior* trees from an Irish provenance trail (sample size (n)=55).

Locus	*No. Alleles**	H_O	H_E	*Gene diversity*	F_{is}
M2-30	79	0.85	0.95	0.89	0.04
FEMSATL 4	72	0.77	0.89	0.87	0.12
FEMSATL 11	60	0.85	0.78	0.78	-0.01
FEMSATL 16	52	0.62	0.75	0.69	0.08
FEMSATL 19	74	0.76	0.91	0.88	0.13
Total	337	0.77	0.86	0.82	0.06

Data obtained from Harbourne (2004). H_O, observed heterozygosity, H_E, expected heterozygosity and F_{is}, inbreeding coefficient. * Approximately equivalent to allelic richness.

The Irish provenance trial

A map showing the locations of where seed was sourced for use in the provenance trail is shown in Figure 6. 2. The data presented in table 6. 21, is for the Irish provenance trial (data from Harbourne, 2004). Samples were collected from trees, grown from seed, sourced in fourteen different locations around Ireland by Coillte and set up in a provenance trial at Drumsna, Co. Cavan. A total of 337 alleles were generated from five microsatellite loci tested in this study (table 6. 21). All five microsatellite loci scored were highly polymorphic, displaying a high number of alleles (from 52 to 79 alleles per locus). The H_O value was lower than the H_E value for four out of five loci resulting in positive values for F_{is}. Only the FEMSATL 11 loci had a higher H_o than H_e resulting in a F_{is} value of 0. The mean F_{is} value for all loci was 0.06 (F_{is} values ranged from 0.0 to 0.13). The positive value of the inbreeding coefficient indicates an overall heterozygote deficiency in the sample.

Three natural Irish populations

The data presented in table 6. 22, is for three natural Irish populations (data from Harbourne, 2004). Samples were collected from trees growing in three natural Irish populations at St. John's Wood, Co. Roscommon, Hanging Rock Wood, Co. Fermanagh and Slieve Carron Wood, Co. Clare. A total of 182 alleles were generated from five microsatellite loci tested in this study (table 6. 22). All five microsatellite loci scored were highly polymorphic, displaying a high number of alleles (from 15 to 60 alleles per locus). The H_O value was lower than the H_E value for all five loci resulting in positive values for F_{is}. The mean F_{is} value for all loci was 0.20 (F_{is} values ranged from 0.17 to 0.39). The positive value of the inbreeding coefficient indicates an overall heterozygote deficiency in the population. Hardy-Weinberg equilibrium (HWE) was rejected for all loci ($p<0.01$), all showed a significant deficiency of heterozygotes.

Table 6. 22. Allelic diversity of five microsatellite loci scored in a sample of three natural Irish populations of *Fraxinus excelsior* (sample size (n) = 154).

Locus	*No. Alleles*	*Allelic richness* **	*Range of sizes (bp)*	H_O	H_E	*Gene diversity*	F_{is}
M2-30	60	37.6	182-353	0.76	0.95	0.95	0.20*
FEMSATL 4	38	24.7	160-237	0.75	0.90	0.90	0.17*
FEMSATL 11	34	23.4	185-240	0.74	0.91	0.91	0.18*
FEMSATL 16	15	10.4	177-210	0.41	0.67	0.67	0.39*
FEMSATL 19	35	23.3	144-221	0.74	0.88	0.88	0.17*
Total	182	-	-	0.68	0.86	0.86	0.20*

Data obtained from Harbourne (2004). H_O, observed heterozygosity, H_E, expected heterozygosity and F_{is}, inbreeding coefficient. * F_{is} is significantly different from Hardy-Weinberg equilibrium (HWE), $p<0.01$. ** Adjusted to sample size n=50.

6. 7. Discussion

The aim of this chapter was to assess the clonal fidelity of vegetatively propagated plants produced by grafting and micropropagation methods. Furthermore, the overall level of genetic variation within the selected ash population was revealed and compared to that found in studies of an Irish provenance trial and three natural Irish populations (data from Harbourne, 2004).

6. 7. 1. Genetic fidelity within genotypes

Polymorphisms were detected between vegetatively propagated clones by all five microsatellite loci tested. From a total of 80 trees propagated by grafting 32.5% were considered true clones (defined as having identical fingerprints), whereas from a total of 32 trees propagated by axillary bud proliferation *in vitro* 50% were considered as true clones. The genomic heterogeneity observed within the grafted clones was most frequently detected at the FEMSATL 16 locus followed by the FEMSATL 4, FEMSATL 19, M2-30 and FEMSATL 11 loci. Variation included polymorphic alleles at the same locus and more commonly plants missing an allele at a heterozygous locus or gaining an allele at a homozygous locus. Length polymorphisms generally differed by more than one base pair and in most cases by between 4 and 20 base pairs. Nevertheless, most of the grafted plants within a particular genotype are grouped together on the NJ tree indicating that they are more similar to each other than to any other individual in the study and that they most likely originate from the same mother tree, using this criterion 90% of the grafted trees can be said to originate from the same mother tree.

The grafted "clones" which are not grouped with the other plants of their supposed genotype and obviously do not originate from the same mother tree may have arisen from numerous sources such as shoots arising from the root stock and the misidentification of plants which can occur at any stage in the process from collecting the scion wood to collecting and processing the DNA samples. The more subtle changes in the DNA patterns between grafted clones may be explained by experimental error, a small amount of which can be expected when scoring bands in a DNA profile. For AFLP analysis of *Miscanthus* clones the experimental error due to band scoring was estimated in the range of 1-2% (Hodkinson *et al.*, 2002). Genotyping errors can also be caused by null alleles, suspected to occur at microsatellite loci (Morand *et al.*, 2002), variation between identical genotypes can be introduced by an allele not amplifying at a heterozygous locus and the sample being recorded as a homozygote. This phenomenon was frequently seen during the scoring of alleles in this study where alleles disappeared and appeared within clones at certain loci (refer to the allele size tables in appendix 6). Morand *et al.* (2002) calculated the frequency of a null allele at each locus using the following method. At a given locus, the frequency p^2 of the individuals that failed to amplify any fragment also represents the frequency of the homozygotes for the supposed null allele. Assuming then Hardy-Weinberg equilibrium the frequency of the null allele, if it exists, should be p. According to the number of non-amplifying individuals per locus, in the analysis of all samples in this study (including both the genetic fidelity study and the sample of the population of selected genotypes). The frequency of a null allele ranged from 0 (FEMSATL 4), 0.08 (FEMSATL 19 and M2-30), 0.11 (FEMSATL 11) to 0.14 (FEMSATL 16). Interestingly, when examining the raw data within the grafted clones, the phenomenon of alleles appearing and disappearing occurs more frequently at the FEMSATL 16 and the FEMSATL 4 loci where, as mentioned in the previous paragraph, variation between clones was most frequently detected. Morand *et al.*, (2002) reported the frequency a null allele in samples from 12 seed stands as between 0.25 (FEMSATL 11) and 0.38 (FEMSATL 19).

Another source of non-detection of microsatellite alleles is the short allele dominance, where in heterozygote alleles individuals that have a short and a long allele, only the short allele is detected and scored (Wattier *et al.*, 1998). In this study, when both homozygotes and heterzygotes were detected within clones at the FEMSATL 16 locus the homozygotes usually only had the shorter allele. Morand *et al.* (2002) did preliminary experiments on short allele dominance, using a 6% polyacrylimide gel found that the long alleles had decreased band intensity compared to short alleles but were still scorable on the gel and were most often detected as a real allele. Sometimes, however, they were identified as a suspected allele. In this study, samples were genotyped using an automated sequencer where the intensity of a band is represented by the height of a peak on the electrophorogram. Only peaks higher than a certain point were scored as alleles, so it is conceivable that real alleles may not have been identified if they were below this point. Allelic dropout can also occur resulting in an allele failing to

amplify due to stochastic sampling error if using low template DNA concentrations (Miller and Watts, 2003). Polymerase error rendering a false allele (Gagneux *et al.*, 1997; Goossens *et al.*, 1998) and amplification of contaminant DNA (Miller *et al.*, 2002) are genotyping errors that occur less frequently at microsatellite loci but cannot be discounted as a source of variation between samples.

Intra-clonal variation can occur from instabilities within the SSR regions stimulated to occur under certain environmental or physiological conditions. Puchta *et al.* (1995) demonstrated that recombination frequencies in somatic cells of *Arabidopsis thialiana* and tobacco plants can be increased by DNA damaging agents such as UV light and in the case of *Arabidopsis thialiana* can be induced by exposure to salt stress. Rare intra-variety polymorphism has been detected in microsatellite loci analysed for *Olea europaea* (Cipriani *et al.*, 2001) and also in RAPD and AFLP analysis of *O. europaea* cultivars propagated through grafting (Weisman *et al.*, 1998; Sensi *et al.*, 2003). Intra-clonal variation was detected in 30-100 year old vegetatively propagated plants of the grapevine cultivar 'Picolit' by SSR and AFLP analysis, in this case the possible causes of variation which included extra alleles was focused on solid mutations and chimaerisms (Zulini *et al.*, 2003).

There is almost conclusive evidence for the occurrence of somatic mutations in plants (Whitham and Slobodchikoff, 1981; Antolin and Strobeck, 1985; Schaal, 1987; Klekowski, 1988; Gill *et al.*, 1995; Kovalchuk *et al.*, 2000b). Within individual variation for polygenic and cytogenetic traits has been documented by several authors (Lewis *et al*, 1971; Klekowski, 1988; Klekowski and Godfrey, 1989). In addition, molecular genetic variation has been detected among naturally occurring clones in several plant species (Schaal and Learn, 1988; Capossela *et al.*, 1992; Tuskan *et al.*, 1996). Such somatic variation is expected to be most common at loci with high mutation rates, as reported for microsatellite DNA sequences (Weber and Wong, 1993; Schlötterer *et al.*, 1998; Udupa and Baum, 2001). There is, however, little information about the stability of microsatellite loci during somatic development of plants (Klekowski, 1988; Smith and Devey, 1994; Gill *et al.*, 1995; Elsik *et al.*, 2000). Most of the polymorphic alleles found in this study differ by at least four repeat units from the parental allele, and thus contradict the expectation that mutations at microsatellite loci should involve single repeat unit changes following slippage during DNA replication (Ellegren, 2000; Schlötterer, 2000). There is little quantitative information available on rates of somatic mutation in plants. Somatic base substitution frequency in plants has been estimated to be two to three orders of magnitude higher than in other organisms (Kovalchuk *et al.*, 2000b). Genomic mutation rates for microsatellite loci in plants have been estimated to fall within the range of 10^{-2} to 10^{-3} for nuclear-encoded loci with tri- and dinucleotide repeats (Kovalchuk *et al.*, 2000a; Udupa and Baum, 2001) to 10^{-5} for chloroplast encoded loci with mononucleotide repeats (Provan *et al.*, 1999). It is important to remember that markers selected for genotyping in diversity studies are likely to be among the most polymorphic regions within a genome

and given the relationship between polymorphism and mutation rate, such loci might be associated with higher mutation rates that the genome average (Ellegren, 2000a).

Graft-induced genetic changes have been reported by several authors including; Ohta (1970, 1977 a, b, c, 1991); Kasahara *et al.* (1973); Ohta and Choung (1975 a, b); Pandey (1976); Yagishita and Hirata (1987) and Yagishita *et al.* (1990). Ohta (1991) lists the three most significant graft-induced genetic changes as follows:

(1) Simultaneous alleles change: Direct changes from dominant homozygous to recessive homozygous, that is, both alleles change at the one time. This can never happen by sexual crossing or by contamination, and must be a very rare mutation. This type of change was obtained by ordinary grafting and has been reported in transformation experiments by applying DNA in insects such as *Ephestia* (Nawa and Yamada, 1967, 1968).

(2) Simultaneous complementary gene change: In the case of two complementary genes, both genes always changed together. No instance was observed of only one gene changing. Kasahara *et al.* (1973) obtained 39 variants on pericarp colour among 1210 individuals in G1 generation from K*/T grafts of red pepper (*Capsicum annuum* L.). Of the 39 variants, 36 were red indicating that both genes changed together, 2 variants were salmon red and one orange which should have been more frequent if the mechanism was a free assortment of independent genes (chromosomes) as in sexual hybridization.

(3) Chimeras produced: the changed states are usually stable in the variants, but sometimes are unstable, causing chimeric mosaicism within a single plant.

Ohta (1991) put forward the following mechanism, of chromatin translocation between the stock plant and the scion, as source of graft-induced genetic change in red pepper. In the plant, metaplasm and protoplasm can translocate from old dying cells in lower withering leaves to the still active primordia, and is utilized as material for new cells. In plants, such as red pepper the effect of translocated chromatin must be quite small due to the small leaf size and the fact that only a few leaves drop before maturation of the fruits and seed (Ohta, 1991). However, heavy lignification in the cells of the stem is a characteristic of red pepper and many tree species, and chromatin in various shapes and sizes, observed in the lignifying cells is considered as the donor DNA or the transforming DNA. No genetic effect is associated with the chromatin translocation in a normal plant, since all the cells consist of an identical genotype. Conversely, in the case of graft combinations, where genotypes are different between the scion and stock, this chromatin translocation observed by Ohta (1991) could have special significance. The fact that neither chromosomes nor naked DNA, but rather chromatin translocates may help to explain the fact that both alleles change together and that two complementary genes change together at one time in red pepper. No transmission of genetic information will take place if DNA molecules of the stock are disintegrated to nucleotides and are utilized by the scion soley as raw

material in the formation of new cell nuclei with its own DNA strand as a template. Thus, the translocation of chromatin masses from the dying cells of the stock to the scion growing point must be a master key to the mechanism of graft-transformation (Ohta, 1991). The mechanism of translocation of chromatin was supported by Pandey (1985), although he also proposed another idea on the origin of transforming DNA, that is, protoplast fusion at the stock-scion interface resulting in chromatin condensation and "pulverization" of stock DNA (stock cells at the S phase and scion cells at the M phase) (Pandey, 1976). Heredity changes similar to those induced by grafting have also been induced by DNA treatment of seeds. Ohta (1991) treated seeds of one pepper cultivar with the DNA from the leaves of a different cultivar, the seeds were germinated and three fruiting characters known to be Mendelian traits were analysed. The changes found in the plants were exactly the same as those induced by grafting experiments, indicating that the gene changes can be induced by either treatment.

The formation of chimeras in the shoot apex can cause polymorphisms to be detected between vegetatively propagated plants. Hansen *et al.* (2004) produced a perclinal *Populus*-chimera (MA), by grafting together *P. x canadensis* 'Marilandica' (M) and *P. maximowizcii x P. trichocarpa* 'Androscoggin' (A). Periclinal chimeras were identified as contributing to polymorphism between clones of *Vitis vinifera* 'Pinot' (Hocquigny *et al.*, 2004). Organs such as leaves have their origins in the shoot apex. If the shoots apex is composed of layers of mutant and normal cells, so too are the organs which arise from the apex (Marcotrigiano, 1991). Chimeras can arise when a mutation occurs in one cell layer of the shoot apical meristem but not in other layers. With time the whole cell layer becomes populated with mutant cells forming a perclinal chimera. The vast majority of variegated-leaf chimeras have arisen by spontaneous nuclear or plastid mutation (Kirk and Tinlney, 1978). However, in the case of the grafted ash trees studied, this scenario is unlikely because you would expect to detect alleles from both genotypes at the microsatellite loci resulting in three or four alleles appearing in the electrophorogram, this did not occur.

As previously mentioned, 50% (16/32) of the *in vitro* propagated plants were considered, after microsatellite analysis, as true clones. There is some evidence of possible somaclonal variation within the clones and there is also substantial evidence that mislabeling of samples has occurred at some stage either in the propagation phase or the during the DNA analysis. Micropropagated plants of genotype F5 in the glasshouse were grouped together on the NJ tree with those still in the *in vitro* propagation phase, indicating that they are all from the same mother tree. Variation between samples of genotype F5 included both a missing allele and a polymorphic allele at the FEMSATL 4 locus and polymorphic alleles at the FEMSATL 19 and M2-30 locus. This may indicate genotyping errors including null alleles, allelic drop out or short allele dominance (discussed previously) or somaclonal variation occurring at these loci. For all the polymorphisms recorded base pair differences were greater

than 1. For genotype 47 all the micropropagated plants, weaned and *in vitro*, were identical but were grouped separately from the grafted plants of this genotype and close to micropropagated plants of genotype 49. The grafted plants of both these genotypes are grouped closely together as expected considering that they were collected from the same population at Castlecomer, Co. Kilkenny. However, one would also expect the grafted plants and micropropagated plants of the same genotype to cluster together on the NJ tree. The fact that this is not occurring suggests that the two groups of plants, grafted and micropropagated plants, are not derived from the same mother tree. The sample C49A from the *in vitro* clones is clearly different from both the other micropropagated plants and grafted plants of genotype 49 and has polymorphisms at four out of five loci, indicating that either this plant is not from genotype 49 or has undergone substantial changes in its DNA. The micropropagated plants of genotype 70 are clustered in a group with those of genotype 72, in fact two of the *in vitro* samples of genotype 70 are considered identical to those of genotype 72. Indicating that a mix up between genotypes had occurred at some stage during micropropagation. However, similarly to the grafted plants, most of the micropropagated plants within a particular genotype are grouped together on the NJ tree indicating that they are more similar to each other than to any other individual in the study and that they most likely originate from the same mother tree, using this criterion 93.7% of the micropropagated plants can be said to originate from the same mother tree.

Variation between propagules in the micropropagation process can be introduced from many sources including from the original material used to initiate a culture. As seen in the results (section 6. 6. 1), not all the grafted plants from the same mother tree are genetically identical and if buds from more than one grafted propagule are selected to initiate a culture line this can introduce variation into that culture line. Somaclonal variation (discussed chapter 1 section 1. 9) may also occur during micropropagation however, by using the system of axillary bud proliferation rather than that of adventitious shoot culture it is expected to be low. Somaclonal variation may result from modifications in the chromosome number or methylation pattern, chromosome breaks, tranposons activation, deletions, genome rearrangements, endopolyploidy, or nucleotide substitutions (Larkin and Scowcroft, 1981; Evans *et al.*, 1984; Deverno *et al.*, 1994; Kawata *et al*; 1995; Jaligot *et al.*, 2000). The species, explant type, and the donor genotype can all affect the variation rate as can the composition of the culture medium, conditions of the physical culture and the duration between successive subcultures (Vasil *et al.*, 1979; Vasil and Vasil, 1980; Ahuja, 1987; Feuser *et al.,* 2003). Feuser *et al.* (2003) identified the temporary immersion system of micropropagating *Ananas comosus* (L.) Merr. to produce the most shoots and the least amount of somaclonal variants compared to a stationary immersion system. The frequency of somaclonal variant detection varies between 0 and 40% in the literature and greatly depends on the marker system used (Shoemaker *et al.,* 1991; Isabel *et al.*, 1993; Skirvin *et al.*, 1994; Barrett *et al.*, 1997). RAPD markers only detected somaclonal variation in one *in vitro* plant of the chestnut hybrid

(*Castanea sativa x C. crenata*) even though the culture line was in *in vitro* multiplication for four years and significant differences were recorded in the growth patterns of the putative clones (Carvalho *et al.*, 2004). Devarumath *et al.* (2002) demonstrated that somaclonal variation occurred in micropropagated tea clones (*Camellia sinensis*) produced by axillary branching from organised meristems and that genomic changes were genotype dependant rather that culture condition dependant. Furthermore, Devarumath *et al.,* (2002) observed that a relatively low level of polymorphism was detected in the nuclear as opposed to the mitochondrial genome and that ISSR fingerprinting detected nearly three times more polymorphic loci than did RAPD fingerprinting. Somaclonal variation was detected by SSR analysis of *Populus tremuloides* micropropagated from dormant vegetative buds even though only small numbers of plants were tested (Rahman and Rajora, 2001). The variation detected in the somaclones included the appearance of a new allele of increased size at one locus hypothesised to be due an addition of the repeat units and in another plant the presence of an extra allele at a different locus presumed to be due to the presence of a single extra chromosome or duplicate of a chromosomal segment. Silveira and Cottignies (1994) found no variation in chromosome number (2n=46) in the root apex of *in vitro* plants acclimitized after 5 months in culture compared two-year-old wild plants of *F. excelsior* growing in the field. There are no reports in the available literature of genetic fidelity tests of vegetatively propagated ash trees using molecular markers. We did not assess chromosome number in this thesis but it would be worth screening the samples with flow-cytometric methods to detect chromosome number change.

6. 7. 2. Genetic diversity within the selected population

The genetic diversity assessed with microsatellite markers in the sample of selected genotypes of *F. excelsior* in this study is relatively high: the average gene diversity over all loci is 0.88 and the observed number of alleles per locus ranges from 11 to 46 (total 141). This compares favourably with results from the three natural Irish populations analysed by Harbourne (2004) (table 6. 22). Average gene diversity from the three populations combined was 0.86 and allelic richness per locus (the number of alleles independent of sample size) ranged from 10.4 to 37.6. The number of alleles per locus in the provenance trial was higher, ranging from 52 to 79 (total 337), and gene diversity estimated at 0.82 was slightly lower compared to the selected and natural populations. All three studies of ash diversity (selected genotypes, provenance trial and natural populations) indicated a general deficiency of heterozygotes in the population by having positive F_{is} values. The values of F_{is} for the selected population and the three natural populations were more similar (0.32 and 0.20) than the F_{is} recorded for the provenance trial material (0.06). It would therefore appear that the diversity of the selected population in terms of allelic number is similar to the natural populations but lower than the provenance trial material. It is therefore less representative than the provenance trial material.

Heuertz *et al.* (2004) used the same five microsatellite loci, to look at the genetic structure of the European population of *F. excelsior,* as were used to study the Irish populations. Data from 36 populations (1069 individuals overall) revealed the total number of alleles per locus ranging between 16 to 83, with an overall total of 275 alleles scored over five loci. The average within-population gene diversity equalled H_E=0.77 and the total gene diversity H_T=0.83. Populations were on average slightly inbred with a mean F_{is} of 0.03. In a study of 10 Bulgarian ash populations using 321 individuals, total gene diversity was estimated at 0.79 and the observed number of alleles, in the same five microsatellite loci as used for the Irish populations, ranged from 10 to 59; a positive F_{is} value of 0.01 was also reported for the Bulgarian populations (Heuertz *et al.*, 2001). Morand *et al.* (2002) reported similar findings in a study of French *F. excelsior* populations, within the seed stage the number of alleles per locus ranged between 36 and 56 and the mean F_{is} value was 0.16 but for the adult trees sampled the F_{is} value was 0.292 (number of alleles per locus was not reported for the adult trees). Heurtz *et al.* (2003) reported a similar heterozygote deficiency in a Romanian population of *F. excelsior.* Similarly the provenance trial set up in Ireland has a lower F_{is} value (F_{is}=0.06) compared to either the selected genotype population (F_{is}=0.32) or the natural populations (F_{is}=0.20), this trial was established from seed sourced throughout Ireland, whereas the other two populations were both sampled from adult trees. As illustrated on the map (figure 6. 2) the selected genotypes sampled for this study are mostly from the same geographic area in the south east of the country whereas the provenance trial material was collected from a broader area. Furthermore, the selected genotypes were non-randomly selected from the natural Irish population, based on the desirable attributes of their phenotype commonly accepted to be a result of environmental and genotypic influences. Morand *et al.* (2002) put forward several explanations for the deficiency of heterozygotes in the French ash population. Firstly the unbiased transmission of alleles was excluded as experiments showed that the microsatellite loci used presented a Mendelian inheritance of alleles. Secondly, the presence of null alleles was suspected, thirdly, short allele dominance (Wattier *et al.*, 1998), however this was proved unlikely after experimentation. Fourthly, the existence of a Walhund effect, due to the presence of inbreeding subunits inside the studied population, and finally excess homozygosity within populations could be explained by mating among relatives, or partial selfing which was found to occur in sporadically in natural European oak populations (Bacilieri *et al.*, 1995; Streiff *et al.*, 1999) and was demonstrated in *F. excelsior* during pollination experiments (Morand-Prieur *et al.*, 2003).

Comparing the data on the genetic diversity of a sample of the population of selected Irish ash genotypes to that of the other European studies on genetic diversity of *F. excelsior* it can be seen that the diversity in the selected population is at a similar level to that of natural ash populations and therefore should contain sufficient diversity for a tree improvement program. However the collection

could be enhanced by adding new material to bring diversity levels in line with the provenance sample (with broad Irish range).

This study has demonstrated the suitability of microsatellite markers to detect variation between vegetative propagules. However, the presence of variation due to null alleles and other genotyping errors was suspected. Re-sampling the variant plants and repeating the procedure from DNA extraction to genotyping may confirm suspected sampling errors. Further investigation of plants with mutant alleles might also include re-sampling and sequencing of the mutant allele(s) to determine the base changes that occurred. Micropropagation techniques were best used to ensure the genotypic fidelity of the propagules, however, mislabelling of cultures and possible somaclonal variation was apparent. Trees produced by grafting techniques appeared to have accumulated slightly more variation within genotypes than the micropropagated plants. The value of these molecular markers for genetic resource characterisation of ash and identification of variant individuals has been elucidated.

CHAPTER 7. Summary

7. 1. Introduction

For conventional forestry purposes, *F. excelsior* is most commonly propagated by seed. Ideally seed should be collected from provenances (sources) which have been shown to be well adapted environmentally and have a high potential for growth and tree quality. For genetic improvement of a tree species, the best trees in the best provenances are selected as parents in uncontrolled crosses to produce improved seed stocks. This is achieved by grafting scions from the selected trees and establishing the grafted plants in a single area where they will interpollinate naturally to form seed orchards. However, seed progeny is heterogeneous, and requires a long period of growing out time before the plus trees can be identified. By identifying mature plus trees (selected trees) and vegetatively propagating them it is possible to make testing more efficient and to gain valuable time in tree breeding programs (Werner, 1996). The vegetatively propagated trees can be used as mother trees in a seed orchard or as crop trees when deployed as a polyclonal variety. The classical method to clone selections of ash is by grafting in spring onto root stocks obtained by sowing seeds of the common ash (Bailey, 1917). Hammatt (1994) was the first to report on micropropagation of adult *F. excelsior* and since then only Hammatt (1997) and Pierik and Sprenkels (1997) have published papers on micropropagation of adult common ash trees. This thesis has improved known methods of micropropagation of ash and developed new ones for mature ash trees. Furthermore, the propagation season for ash has been extended to include the late winter period by the development of a forcing system to produce shoots from dormant winter buds and by demonstrating the root ability of dormant shoots from previously micropropagated plants. The suitability of microsatellite markers for genetic fidelity testing in vegetatively propagated ash also has been demonstrated.

7. 2. Winter dormancy breaking

Previously ash cultures from mature trees were only initiated from actively growing shoots (Hammatt, 1994; 1997; Pierik and Sprenkels, 1997). This meant that the propagation season was confined to a short time period during spring/ early summer. The results in chapter 2 of this thesis show that it is possible to harvest stem segments during winter dormancy in February, store them at 4°C for at least one month and induce bud burst by applying a warm water treatment (37°C for one hour) followed by forcing under lights in a greenhouse. Shoots produced from buds given the forcing treatments were used to initiate ash cultures *in vitro*. The explants produced by this forcing system were relatively free from contaminants, a factor cited by many researchers as the main obstacle to overcome when initiating cultures from mature trees (Skirvin *et al.*, 1986; Hammatt, 1994; 1997; Pierik and Sprenkels, 1997). Although no proliferating shoot cultures were established using this method it is expected that this is achievable in the future. Experiments on the harvest time

and conditions for forcing of dormant stems may improve the method. Testing the collection time of the new shoots after forcing may also improve the method. Furthermore, a large number of genotypes should be tested to see if there is variation within the species to these forcing treatments and the chance of obtaining proliferating shoot cultures. Initiating cultures from shoots produced by forced dormant stems has been successful in other species of *Fraxinus* including *F. angustifolia* (Perez-Parron *et al.*, 1994) and also in *Malus x domestica*, *Castanea* spp., *Fagus sylvatica* and *Quercus robur* (Skirvin *et al.*, 1986; Vieitiz *et al.*, 1986; Chalupa, 1996; Marks *et al.,* 1987). Shoots produced by the forcing method can be used in other applications such as the genetic diversity study in chapter 6 of this thesis which used leaves collected from the forced shoots for DNA extraction and analysis.

7. 3. Establishing ash shoot cultures *in vitro*

The results in chapter 3 of this thesis highlight the difficulties associated with establishing shoot cultures from mature trees. Initiating dormant ash buds *in vitro* directly after heat treatment successfully reduced contamination and in many cases stimulated bud burst. However it failed to yield any proliferating cultures. The main obstacle was browning of the explant tissue and surrounding media, which was reduced by the addition of the antioxidant glutathione (5mg/l) to the culture media. Silveira and Cottignies (1994) successfully initiated ash cultures from heat treated dormant winter buds of ash proving that it is possible. The results of this thesis show that the optimum time of year to harvest buds for culture initiation was in spring when the buds were flushing naturally. The contamination rates were low and explant browning was less problematic than later in the growing season. Nodes, from naturally flushing shoots, were more viable than shoot tips *in vitro* during the initiation stage and a basal WPM medium with no plant growth regulators or antioxidants was the optimum initiation medium. The inclusion of glutathione to the initiation medium was not tested on the actively growing buds; however this may be interesting to test in future studies on ash. Ruigini (1984) showed that a drop of glutathione solution placed on *O. europaea* callus reduced browning and Nomura *et al.* (1998) used a glutathione dip treatment to prevent browning of shoot tip explants of apple (*Malus pumila*) at the initial stage of culture. The most viable source of explants for initiating ash cultures *in vitro* were previously micropropagated plants growing in the greenhouse. Even though explants were harvested from these plants late in the season viability levels were over 66% and proliferating cultures were established for each genotype tested. This may indicate that the plants were reinvigorated by the tissue culture process. Both Hammatt (1994) and Pierik and Sprenkels (1997) report the apparent reinvigoration of ash cultures from mature trees resulting in the increasing ability of shoots to root *in vitro* after a number of subcultures. A conclusion further supported in this thesis by the ability of cuttings taken from micropropagated weaned plants to root even without auxin treatment (chapter 5). Many studies have demonstrated that culture initiation is more successful from material displaying juvenile

characteristics (e.g. epicormic shoots and stump sprouts) compared mature tissues (Vieitez *et al.*, 1985; Vieitez *et al.*, 1986; Meier-Dinkel, 1987; San-Jose *et al.*, 1988; Chalupa, 1993).

7. 3. 1. The genotype effect

The influence of genotype as affecting culture initiation was observed throughout the experiments (chapters 2 - 5). Explants taken from plants weaned from the micropropagation process were easily established *in vitro.* The fact that the weaned plants had previously been successfully initiated *in vitro,* from mature trees, may demonstrate that they are more amenable to the micropropagation process. It was noted in the laboratory in Teagasc, Kinsealy that different workers initiating a variety of genotypes frequently managed to establish the same genotypes even when collected from different sources (shoots from forced dormant stems, buds from grafted plants in the field and from grafted plants in the greenhouse). The genotype effect during culture establishment has been reported for many species including *Eucalyptus marginata*, *Prunus* spp. and *Quercus petraea* (Hammerschlag, 1982; San-Jose *et al.*, 1990). The genotype effect on culture performance was observed in other stages of the micropropagation protocol, during shoot production and rooting (chapters 4 and 5).

7. 4. Shoot production

7. 4. 1. Shoot culture regime and polyamine metabolism

In chapter 4, the concentration of polyamines, known indicators of abiotic stress in plants (Piqueras *et al.*, 2002) were measured from shoots cultured in three regimes. This experiment was designed to assess the suitability for ash micropropagation of continuous culture on a high salt medium containing plant growth regulators (M91) compared to alternating between M91 medium and the low salt medium with no plant growth regulators (QRC) containing activated charcoal. An increase in the levels of putrescine in shoots cultured continuously on the M91 medium was frequently correlated with a decline in propagation rate. A decline in plant health was also observed in many of these shoots in the form of physiological disorders such as shoot fasication and vitrification of the leaves. Different responses, measured as a change in polyamine concentration, were recorded between the four genotypes of ash tested. An increase in spermidine and spermine was observed in the stress tolerant genotype F5 cultured continuously on M91 medium whereas no increase in these two polyamines occurred in the more sensitive genotype S11. Bouchereau *et al.* (1999) suggested stress tolerance is conferred to genotypes having the ability to transform putrescine into the higher molecular weight polyamines (spermidine and spermine). This is an adaptive response to stress not seen in susceptible organisms. Both spermidine and spermine have been shown to have protective functions in the plant cells and can act as anti-senescent compounds in response to stress, while putrescine produces

either no effects or negative effects, such as depolarization of membranes, potassium leakage, tissue necrosis, and protein loss (Popovic *et al.*, 1979; Drolet *et al.*, 1986; Tiburcio *et al.*, 1990; Kushad and Dumbroff, 1991; Davies *et al.*, 1991; Besford *et al.*, 1993; Bratton, 1994; Bar *et al.*, 1996; Benavides *et al.*, 1997; Bouchereau *et al.*, 1999).

This new information, with respect to the polyamine production of *F. excelsior* shoot cultures *in vitro*, lends further support to the idea that particular genotypes are more suited to the tissue culture process. Furthermore, it was shown that the optimum culture regime for micropropagation of ash shoots was to culture alternately on medium containing plant growth regulators and on QRC medium with charcoal. Shoots produced by this system were healthy, free from physiological disorders and produced little callus minimising the opportunity for adventitious shoot production, hence decreasing the risk of introducing unwanted somaclonal variation into the culture lines. This system has previously been used in micropropagation of *Quercus robur* and *Prunus persica* to promote shoot elongation and overcome the negative effect of cytokinin in the medium (Jona and Vigliocco, 1985; Favre and Juncker, 1987).

7. 4. 2. Bacterial contamination in vitro.

Shoot cultures of ash in the study often showed the presence of bacterial contaminants. These were found either around the base of the shoot explant and/ or in the medium surrounding the explant. Many plants have an endogenous flora of bacteria, viruses and mycoplasmas (Meier and Reuther, 1994; Penlaver *et al.*, 1994; Niemirowicz-Szczytt *et al.*, 2000). Many researchers of woody plants have reported the outgrowth of micro-organisms during *in vitro* culture including those working on ash (Hammatt, 1994, 1997; Peirik and Sprenkels, 1997). In some cases the bacteria have been identified (Penlaver *et al.*, 1994) but in others, as in ash the bacteria have not been fully identified (Hammatt, 1994, 1997; Peirik and Sprenkels, 1997). Recent studies have indicated that a red-pigmented methylotrophic bacterium (*Methylovorus mays*) has a positive effect on plant growth through increased cytokinin production from either the bacterium itself or through stimulation of the plant (Kalyaeva *et al.*, 2001, 2003). In the studies in this thesis it was observed that stable shoot cultures which were transferred regularly did not show signs of bacterial contamination and the bacteria and plant may be considered to be in a homeostatic state. It was noted that the outgrowth of bacteria was most common in explants cultured continuously on medium with high levels of cytokinin and nutrient salts, during rooting and also when small sized explants were used. It is thought that in the above conditions the tissues are in a stressed physiological state which favours membrane leakage (Tiburcio *et al.*, 1990). The leakage of nutrients from the cells may be the trigger for bacteria to proliferate. The results chapter 4 of this thesis showed that increasing stress

levels in ash (indicated by an increase in putrescine concentration) were correlated with an increase in bacterial contamination.

7. 4. 3. *Shoot production medium*

The results of experiments performed in chapter 4 of this thesis have identified modified olive medium (OM) as the optimum medium on which to culture ash shoots when compared to modified MS, modified WPM and DKW. Olive medium was formulated following the analysis of the mineral elements in embryos and young shoots of *Olea europaea* found to be characterised by a high content of Ca, Mg, S, Cu and Zn hence, the OM proliferation medium was formulated with higher levels of these elements compared to MS, WPM or DKW. Furthermore, the vitamins folic acid and biotin are included in OM medium but are absent from the other three media formulations tested. Biotin is an essential cofactor for a variety of carboxylase and decarboxylase reactions and is involved in diverse metabolic pathways of all organisms (Wood and Kumar, 1985). Folic acid has been shown to have a growth-stimulatory effect on *Helianthus tuberosus* L. tissues *in vitro* (de Capite, 1952). This is the first report of OM being used to culture mature *F. excelsior* shoots *in vitro*, however it has previously been used for other Oleaceae members including *Olea europaea* (Ruigini, 1984), *F. ornus* (Arrillaga *et al* 1992) and *Phillyrea latifolia* (Lucchesini and Mensuali-Sodi, 2004).

Sucrose (87.6mM) alone or in an equimolar mixture with mannitol, was identified as the optimum carbohydrate to support the growth of ash shoots *in vitro* compared to glucose or mannitol alone. Sucrose has been the major carbohydrate in use for tissue culture purposes and has been commonly used for *in vitro* propagation of *F. excelsior* (Chalupa, 1990; Hammatt and Ridout, 1992; Silveira and Cottignies, 1994; Pierik and Sprenkels, 1997). Mannitol occurs in most members of Oleaceae, where it comprises a significant proportion of the soluble carbohydrate pool (Stoop *et al.,* 1996; Peltier *et al.*, 1997). In tissue culture mannitol has been used as an osmotic agent (Leva and Muleo, 1993), used instead of sucrose in the *in vitro* culture of *O. europaea* (Leva *et al.,* 1994) and more recently for *in vitro* culture of *Phillyrea latifolia* (Lucchesini and Mensuali-Sodi, 2004). A high concentration of the cytokinin BAP (5mg/l) in combination with TDZ (0.25-0.5mg/l) produced high propagation rates (2.2 – 4.6) from nodal explants. Previous research on *Fraxinus* species has demonstrated that BAP and TDZ are effective plant growth regulators for axillary shoot proliferation (Navarrette *et al.*, 1989; Chalupa, 1990). High concentrations of BAP (1 – 5mg/l) have previously been shown to stimulate shoot proliferation in juvenile and mature explants of *F. excelsior* (Chalupa, 1990; Leforestier *et al.*, 1990; Hammatt and Ridout, 1992; Hammatt, 1994; Silveira and Cottignies, 1994).

An interesting study in the future might involve analysing the minerals contained in ash leaves *in vivo,* adjusting the type and concentration of nutrients in the culture medium according to those found naturally in the plant and comparing this new "ash medium" to the standard culture media formulations such as MS, WPM, DKW and OM. This method was previously used by Ruigini (1984) to develop olive medium for culturing *O. europaea* and by Nas and Read (2004) to develop a medium for culturing *Corylus avellana.* In both these studies, an improvement in culture health and an increase in propagation rate was recorded on the new media (Ruigini, 1984; Nas and Read, 2004). Furthermore, this type of culture medium development avoids factorial treatments, thus is less labour intensive, requires fewer explants and reduces the size of experiments necessary for development of a defined tissue culture medium.

7. 5. Root induction and plant growth

This thesis has shown that auxin has a significant effect on rooting in ash; auxins are required for rooting during the induction phase, but can inhibit the process during the formation phase (chapter 5). The optimum treatment for root induction from shoots of *F. excelsior* produced in the shoot production phase of micropropagation was identified as a pulse treatment on auxin containing media in darkness, followed by transfer to auxin free medium containing activated charcoal and subsequent culture in the light. Hammatt and Ridout (1992) rooted shoots of *F. excelsior* by using a pulse treatment on IBA containing medium for three weeks followed by transfer to medium without IBA. Pierik and Sprenkels (1997) showed that a four day dark period after the start of root initiation was required to give high rates of rooting in mature *F. excelsior.* In many plants, the induction of roots is promoted by darkness or light of low fluence, apparently because natural auxin levels increase in these conditions, whereas they are decreased by light of high fluence (George, 1996). The *in vitro* rooting of ash shoots was dependent not only on the genotype of the explant but also on the physiological state of the shoots at the time of experimentation. For example, in experiment 4 (section 5. 6) 21.3% of shoots of genotype 47 rooted compared to 67.2% rooting in experiment 5. Different authors have postulated that the ontogenic stage of shoots within genotypes can affect the rooting capacity through interactions between endogenous and exogenous auxin and differential gene expression (Woo, Hackett and Das, 1994; Marks, 1996; Greenwood *et al.*, 1997; Ballester *et al.*, 1999). Puente and Marín (1997) concluded that differences in the rooting behaviour of an individual *Malus* X *domestica* Borkh. clone were due to changes in the short term physiological state of the shoots.

In this thesis, over 80% of the rooted plantlets were successfully weaned to the glasshouse and it was demonstrated that they can survive outdoors over winter. Weaned plants benefited from the winter chilling by resuming growth earlier than plantlets kept over winter

in a heated glasshouse. It was also demonstrated that 24.7% of ash shoots transferred to *ex vitro* conditions without roots were able to subsequently produce roots and survive weaning to the glasshouse. Indicating that in the future it would be possible to develop a protocol to root ash shoots *ex vitro*. The benefits of which would include a reduction in labour, time and costs associated with micropropagation.

This thesis has demonstrated that weaned plants from the micropropagation process can be used as a source of cuttings for adventitious rooting (chapter 5). Dormant cuttings from previously micropropagated mother plants were induced to form roots, when placed on a heated bench under plastic in the greenhouse, even with no external auxin supply. Previous experiments showed that cuttings collected during the growing season from the same micropropagated stock plants rooted and that cuttings could be harvested three times during the growing season from these stock plants (Douglas, personal communication). Therefore, from a stock of micropropagated plants it is possible to harvest cuttings for rooting four times in one year potentially producing large numbers of propagules for use in field trials, farm demonstrations and seed orchards.

7. 6. Genetic fidelity and diversity tests

It must be ensured that during the vegetative propagation of trees within the selected genotypes the genetic fidelity of those trees is retained. At all stages of the propagation cycle, whether through grafting, micropropagation techniques or rooting cuttings, it is of utmost importance to correctly assign genotypes so mix-ups and mislabelling of plants can be identified. Furthermore, grafted trees must be regularly checked so that the shoots produced and used for propagation purposes are from the scion wood and not from suckers produced by the rootstock. During the micropropagation cycle efforts must be made to reduce the level of somaclonal variation by ensuring that only shoots from preformed meristems are propagated and those arising from callus are discarded. Chapter 6 of this thesis demonstrated the suitability of microsatellite markers in assessing the genotypic fidelity of grafted and micropropagated ash plants. Micropropagation techniques were identified as best used to ensure the genotypic fidelity of the propagules, however, mislabelling of cultures and possible somaclonal variation was apparent. For example, only two out of seven micropropagated plants of genotype F5 were considered genetically identical however, all plants of this genotype grouped together on the neighbour joining tree (figure 6. 1), indicating that they are from the same mother tree but have accumulated slight differences in their DNA profiles. Some micropropagated plants of genotype 70 and 72 were identified as genetically identical, indicating that a mix up between genotypes has occurred at some stage during micropropagation. Trees produced by grafting techniques appeared to have accumulated slightly more variation within genotypes than the micropropagated plants. Variation included polymorphic alleles at the same locus and more

commonly plants missing an allele at a heterozygous locus or gaining an allele at a homozygous locus. However, the extent of this variation that is due to marker related problems such as the presence of null alleles has yet to be determined. Future studies might involve re-sampling the variant plants and repeating the procedure from DNA extraction to genotyping to confirm suspected sampling errors. Further investigation of plants with mutant alleles might also include re-sampling and sequencing of the mutant allele(s) to determine the changes that occurred.

Furthermore, the level of genetic diversity in a sample of the selected trees was elucidated using microsatellite markers and observed to be high. The average gene diversity over all loci was 0.88 and the observed number of alleles per locus ranged from 11 to 46 (total 141). This compared favourably with results from the three natural Irish populations analysed by Harbourne (2004) where the average gene diversity from the three populations combined was 0.86 and total allele number was 182 the number of alleles per locus ranged from 15 – 60. The allelic diversity was much less than that recorded in a sample of provenance material taken from a broad geographic range (337 alleles). It may be therefore advantageous to improve the breeding stock to bring diversity measures of the breeding (selected) population in line with this provenance sample.

This thesis has reported the micropropagation of *F. excelsior* from phenotypically selected mature Irish ash trees from a range of genotypes (chapters 3 - 5), evaluated the genetic fidelity of grafted and micropropagated propagules and evaluated the genetic diversity in a sample of the selected ash trees (chapter 6). A facility for vegetative propagation of selected ash trees can accelerate the genetic improvement of planting stock either through the sexual route where propagated plants form a seed producing orchard or where they can be deployed as a polyclonal variety with high production and quality potential. Furthermore, by developing a method to induce bud burst from forced stems containing dormant winter buds (chapter 2) and to induce rhizogenesis from dormant stems collected from weaned micropropagated plants (chapter 5), the propagation season for ash has been extended, to include the late winter period through to summer. Efficient vegetative propagation of ash by micropropagation and by cuttings has provided plants for field testing so that superior selections can be identified in replicated clonal field trials. Ideally, at least 30-50 selections should be field tested and evaluated for characteristics such as late flushing, growth rate, disease resistance and stem form. The identification of approximately 10 – 15 superior clones can then lead to their deployment as a polyclonal variety (Douglas, personal communication).

References

Abdul-Baki, A. A. (1974). Hypochlorite and Tissue Sterilisation. *Planta* **115**: 373-376.

Adriani, M., Piccioni, E. and Standardi, A. (2000). Effect of different treatments on the conversion of "Hayward" kiwifriut synhtetic seeds to whole plants following encapsultation of in vitro derived buds. *New Zeal. J. Crop. Hort.* **28**(1) 59-67.

Aeschbacher, R., Schiefelbein, J. and Benfey, P. (1994). The genetic and molecular basis of root development. *Ann. Rev. Plant Phys.* **45**: 25-45.

Ahuja, M. R. (1987). Somaclonal variation. J. M. Bonja and D. J. Durzan. *Cell and Tissue Culture in Forestry.* Dordrecht, Martinus Nijhoff Publishers: 272-285.

Ainsworth, E. A., Tranel, P. J., Drake, B. G. and Long, S. P. (2003). The clonal stucture of *Quercus geminata* revealed by conserved microsatellite loci. *Mol. Ecol.* **12**: 527-532.

Albrecht, C. (1986). Opimization of tissue culture media. *Comb. Proc. Int. Plant. Prop. Soc. 1985.* **35**: 196-199.

Altamura, M., Torrigiani, P., Falasca, G., Rossini, P. and Bagni, N. (1993). Morpho-functional gradients in superficial and deep tissues along tobacco stem: polyamine levels, biosynthesis and oxidation and organogenesis *in vitro*. *J. Plant Physiol.* **142**: 543-551.

Altman, A., Kuar-Sawhney, R. and Galston, A. W. (1977). Stabilisation of oat leaf protoplasts through polyamine-mediated inhibition of senescence. *Plant Physiol.* **60**: 570-574.

Alvarez, R., Nissen, S. and Sutter, E. (1989). Relationship between indole-3-acetic acid levels in apple (*Malus pumila* Mill.) rootstocks cultured *in vitro* and adventitious root formation in the presence of indole-3-butyric acid. *Physiol. Plant.* **89**: 439-443.

Al-Zahim, M. A., Ford-Llyod, B. V. and Newbury, H. J. (1999). Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. *Plant Cell. Rep.* **18**: 473-477.

Ameglio, T. (1994). Fonctionnement hydrique du noyer durant la periode non feuillee: consequences sur le developpement de l'arbre. *Seminaire du Groupe d'Etude de l'Arbre 'L'eau dans la vie de l'arbre. Clermont-Ferrand*: 99-129.

Amos, W. e. a. (1996). Microsatellites show mutational bias and heterozygote instability. *Nat. Genet.* **13**: 390-391.

Amzallag, G., Lerner, H. and Poljakoff-Mayber, A. (1992). Interaction between mineral nutrients, cytokinin and gibberelic acid during growth of sorghum at high NaCl salinity. *J. Exp. Bot.* **43**: 81-87.

Anderson, J. V., Chao, W. S. and Horvath, D. P. (2001). A current review on the regulation of dormancy in vegetative buds. *Weed Science* **49**(5): 581–589.

Antolin, M. F. and Strobeck, C. (1985). The population genetics of somatic mutation in plants. *Am. Nat.* **126**: 52-62.

Arencibia, A. D., Carmona, E. R., Cornide, M. T., Castiglione, S., O' Relly, J., Chinea, A., Oramas, P. and Sala, F. (1999). Somaclonal variation in insect-resistant transgenic surgarcane (*Saccharum hybrid*) plants produced by electroporation. *Trans. Res.* **8**: 349-360.

Arndt, F. R., Rusch, R., Stillfried, H. V., Hanisch, B. and Martin, W. C. (1976). SN 49537, A new defoliant. *Plant Physiol.* **57**: 99.

Arrillaga, I., Lerma, V. and Segura, J. (1992). Micropropagation of juvenile and adult Flowering Ash. *J. Amer. Soc. Hort. Sci.* **117**(2): 346
350.

Aue, H. L., Lecomte, I. and Petel, G. (2000). Changes in parameters of the plasmalemma ATPase during peach vegetative bud dormancy. *Biol. Plant.* **43**(1): 25–29.

Bacilieri, R., Ducousso, A., Kremer, A. (1995). Genetic, morphological, ecological and phonological differentiation between *Quercus petraea* (Matt.) Liebl. & *Quercus robur* L. in a mixed stand North-west of France. *Sil. Gen.* **44**: 1-10.

Bagni, N. (1989). Polyamines in plant growth and development. U. Bachrach and Y. M. Heimer. *The Physiology of Polyamines. Vol. 2.* Boca Raton, Florida, CRC Press Inc.: 107-120.

Bagni, N., Barbieri, P. and Torrigiani, P. (1983). Polyamine titer and biosynthetic enzymes during tuber formation of *Heliantus tuberosus*. *J. Plant. Growth. Regul.* **2**: 177-184.

Bagni, N. and Biondi, S. (1987). Polyamines. J. M. Bonga and D. J. Durzan. *Cell and Tissue Culture in Forestry*. Dordrect, Martinus Nijhoff: 113-124.

Bailey, L. H. (1917). The standard cyclopedia of horticulture. New York, MacMillan.

Baker, K. F. (1962a). Thermotherapy of planting material. *Phytopathology* **52**: 1244-1255.

Baker, K. F. (1962b). Principles of heat treatment of soil and planting material. *J. Aus. Inst. Ag. Sci.* **28**: 118-126.

Ballester, A., Sánchez, M. C., San-José, M. C., Vieitez, F. J. and Vieitez, A. M. (1990). Developemant of rejuvenation methods for *in vitro* establishement, multiplication and rooting of mature trees. R. Rodríguez, T. R. Sánchez and D. J. Durzan. *Plant Aging: Basic and Applied Approaches*. New York, Plenum Press: 43-49.

Balloux, F. e. a. (1998). Microsatellite conservation, polymorphism, and GC content in shrews of the genus Sorex (Insectivora, Mammalia). *Mol. Biol. Evol.* **15**: 473-475.

Bangerth, F. (1994). Response of cytokinin concnetration in the xylem exudate of bean (Phaseolus vulgaris L.) plants to decapitation and auxin treatment, and relationship to apical dominance. *Planta* **194**: 439-442.

Bar, Y., Apelbaun, A., Kafkafi, U. and Goren, R. (1996). Polyamines in chloridestressed *Citrus* plants: alleviation of stress by nitrate supplementation via irrigation water. *J. Amer. Soc. Hort. Sci.* **121**: 507–513.

Baraldi, R., Bertazza, G., Bregoli, A., Fasolo, F., Rotondi, A., Predieri, S., Serafini-Fracassini, D., Slovin, J. and Cohen, J. (1995). Auxins and polyamines in relation to differential in vitro root induction on microcuttings of two pear cultivars. *J. Plant. Growth. Regul.* **14**: 49-59.

Barrett, C., Lefort, F. and Douglas, G. C. (1997). Genetic characterisation of oak seedlings, epicormic, crown and micropropagated shoots from mature trees by RAPD and microsatellite PCR. *Scientia Hort.* **70**: 319-330.

Bates, S., Preece, J. E., Navarrete, N. E., Van Sambeek, J. W. and Gaffney, G. R. (1992). Thidiazuron stimulates shoot organogenesis and somatic embryogenesis in white ash (*Fraxinus americana* L.). *Plant Cell. Tiss. Org. Cult.* **31**: 21-29.

Beauchesne, G. and Poulin, C. (1972). La culture in vitro des mèristème, ses possibilitiès et ses limites actuelles pour les plantes ligneuses. P. Chouard and N. de Bilolerling. *Phytotronique et prospective horticole.* Paris, Gauthier-Villars.: 219-231.

Bellarosa, R. (1989). Oak (*Quercus* spp.). Y. P. S. Bajaj. *Biotechnology in Agriculture and Forestry. Trees 2.* Berlin, Springer-Verlag. **5:** 387-401.

Benavides, M. P., Aizencang, G. and Tomaro, M. L. (1997). Polyamines in *Helianthus annuus* L. during germination under salt stress. *J. Plant Growth Regul.* **16**: 205–211.

Ben-Jaacov, J., Ackerman, A., Tal, E. and Jacobs, G. (1991). Vegetative propagation of *Alberta magna* by tissue culture and grafting. *HortScience* **26**: 74.

Bennett, I., McComb, J., Tonkin, C. and McDavid, D. (1994). Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. *Ann. Bot.* **74**: 53-58.

Bergervoet, J. H. W., Jing, H.-C., van den Hout, J. W. E., Delmondez de Castro, R., Kunneman, B. P. A. M., Bino, R. J. and Groot, S. P. C. (1999). Experession of *beta*-tublin during dormancy induction and release in apical and axillary buds of five woody species. *Physiol. Plant.* **106**: 238-245.

Berthon, J., Maldiney, R., Sotta, B., Gaspar, T. and Boyer, B. (1989). Endogenous levels of plant hormones during the course of adventitious rooting in cuttings of *Sequoiadendron giganteum* (Lindl.) *in vitro*. *Biochem. Physiol. Pflanzen* **184**: 405-412.

Besford, R. T., Richardson, C. M., Campos, J. L. and Tiburcio, A. F. (1993). Effect of polyamines on stabilisation of molecular complexes of thylakoid membranes of osmotically stressed oat leaves. *Planta* **189**: 201-206.

Beveridge, C. A., Symons, G. M. and Turnbull, C. G. N. (2000). Auxin inhibition of decapitation induced ranching is dependent on graft-transmissible signals regulated by genes Rms1 and Rms2. *Plant Physiol.* **123**(2): 689–697.

Beveridge, C. A., Weller, J. L., Singer, S. R. and Hofe, r. J. M. I. (2003). Axillary meristem development. budding relationships between networks controlling flowering, branching, and photoperiod responsiveness. *Plant Physiol.* **131**(3): 927–934.

Bhojwani, S. S., Dhawan, V. and Arora, R. (1986). Plant Tissue Culture, a classified bibliography. Amsterdam, Elsevier.

Bino, R. J., Bergervoet, J. H. W., de Vos, C. H. R., Kraak, H. L., Lanteri, S., van der Burg, W. J. and Zheng, X. Y. (1996). Comparison of nuclear replication activity and protein expression patterns during tomato seed germination. *Field Crops Res.* **45**: 71-77.

Biondi, S. and Thorpe, T. A. (1982) Growth regulator effects, metabolite changes, and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. Bot. Gaz. **143**: 20-25

Bisbis, B., Kevers, C. and Gaspar, T. (1997). Atypical TCA cycle and replenishment in a nonphotosynthetic fully habituated sugarbeet callus overproducing polyamines. *Plant Physiol. Biochem.* **35**: 363-368.

Black , M. (1952). The problem of prolonged rest in deciduous fruit trees. *Proc. 13th Intern. Hort. Congress. Vol 2.* London: 1122-1131.

Blakesley, D. (1984). Rooting of cuttings of woody plants. Leister Polytechnic.

Blakesley, D. (1994). Auxin metabolism and adventitious root formation. T. D. Davis and B. E. Hassig. *Biology of Adventitious Root Formation (Basic Life Sciences 62).* New York, Plenum Press: 143-154.

Blakesley, D., Weston, G. and Hall, J. (1991a). The role of endogenous auxin in root initiation. Part 1: Evidence from studies on auxin application, and analysis of endogenous levels. *Plant Growth Regul.* **10**: 341-353.

Blakesley, D., Weston, G. and Elliott, M. (1991b). Endogenous levels of indole-3-acetic acid and abscisic acid during the rooting of *Cotinus coggygria* cuttings taken at different times of the year. *Plant Growth Regul.***1 0**: 1-12.

Boerner, A., Chebotar, S. and Korzun, V. (2000). Molecular characterization of the genetic integrity of wheat (*Triticum aestivum* L.) germplasm after long-term maintenance. *Theor. Appl. Gen.* **100**(3-4): 494-497.

Boget, N., Torne, J. M., Willdino, L. and Santos, M. (1995). Variation in endogenous polyamine content of maize calli obtained from zygotic and androgenetic embryos. *Plant Cell. Tiss. Org. Cult.* **40**: 139-144.

Bon, M. C. and Monteuuis, O. (1991). Rejuvenation of a 100 year old *Sequoiadendron giganteum* through *in vitro* meristem culture. 2. Biochemical arguments. *Physiol. Plant.* **81**: 116-120.

Bonga, J. M. (1987). Clonal propagation of mature trees: problems and possible solutions. J. M. Bonga and D. J. Durzan. *Cell and Tissue Culture in Forestry*. Dordrect, Martinus Nijhoff. **1:** 249 - 271.

Bonga, J. M. and Durzan, D. J. (1982). Tissue Culture in Forestry. The Hague, M. Nijhoff and W. Junk.

Bonga, J. M. and von Aderkas, P. (1993). Rejuvenation of tissues from mature conifers and its implications for propagation in vitro. M. R. Ahuja and W. J. Libby. *Clonal Forestry I. Genetics and biotechnology*. Berlin., Springer-Verlag**:** 182-199.

Borchert , R. (1991). Growth periodicity and dormancy. A. S. Raghavendra. *Physiology of Trees*. New York, John Wiley**:** 221–245.

Boresch, L. (1928). Fruhtreibende Wirkung des Warmbades III. *Biochem. Z.* **202**: 180-201.

Borkowska, B. (2001). Morphological and physiological characteristics of micropropagated strawberry plants rooted *in vitro* or *ex vitro*. *Scientia Hort.* **89**: 195-206.

Botstein, D., White, R. L., Skolnick, M. and Davis, R. W. (1980). Construction of a genetic linkage map using Restriction Fragment Length Polymorphisms. *Am. J. Hum. Genet.* **32**: 314-331.

Bouchereau, A., Aziz, A., Larher, F. and Martin-Tanguy, J. (1999). Polyamines and environmental challenges: recent development. *Plant Sci.* **140**: 103-125.

Bouman, H. and De Klerk, G. J. (2001). Measurement of the extent of somaclonal variation in begonia plants regenerated under various conditions; Comparison of three assays. *Theor. Appl. Genet.* **102**: 111-117.

Bouriquet, R., Tsogas, M. and Blaselle, A. (1984). Essais de rajeunissement de l'épicéa par les cytokinines. *AFOCEL Annales de Recherches Sylvicoles*: 173-185.

Bowen, H. J. M. (1979). Plants and the Chemical Elements. London, Academic Press.

Boxus, P. and Quoirin, M. (1974). La culture de meristemes apicaux de quelques especes de *Prunus*. *Bull. Soc. Bot. Belg.* **107**: 91–101.

Boxus, P. and Terzi, J. (1988). Control of accidental contaminations during mass propagation schemes. *Acta Hort.* **212**: 91-93.

Brachet, S., Jubier, M. F., Richard, M., Jung-Muller, B. and Frascaria-Lacoste, N. (1999). Rapid detection of microsatellite loci in the common ash *Fraxinus excelsior* using 5' anchored PCR. *Mol. Ecol.* **8**: 160-163.

Bradley, U. (1999). Molecular variation in ecologically contrasting populations of *Ranunculus repens*. B.A. Mod. Thesis, Department of Botany. Dublin, University of Dublin.

Brand, M. H. and Lineberger, R. D. (1992). *In vitro* regeneration of *Betula* (Betulaceae): Biochemical evaluation. *Am. J. Bot.* **79**: 618 - 625.

Bratton, D. L. (1994). Polyamine inhibition of transbilayer movement of plasma membrane phospholipids in the erythrocyte ghost. *J. Biol. Chem.* **269**: 22517-22523.

Brearley, J., Henshaw, G. G., Davey, C., Taylor, N. J. and Blakesley, D. (1995). Cryopreservation of *Fraxinus excelsior* L. zygotic embryos. *Cryo-Letters* **16**: 215 - 218.

Brinkmann, B. e. a. (1998). Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat. *Am. J. Hum. Genet.* **62**: 1408-1415.

Brock, G. J. R. e. a. (1999). *Cis*-acting modifiers of expanded CAG/CTG triplet repeat expandability: associations with flanking GC content and proximity to CpG islands. *Hum. Mol. Genet.* **8**: 1061-1067.

Brown, A. D. and Simpson, J. R. (1972). Water relations of sugar-tolerant yeasts: the role of intercellular polyols. *J. Gen. Microbiol.* **72**: 589-591.

Brown, P. H. and Hu, H. (1996). Phloem mobilitv of boron is species dependent: Evidence for phloem mobility in sorbitol-rich species. *Ann. Bot.* **77**: 497-505.

Browne, R. and Hicks, G. (1983). Developement *in vitro* of white ash buds. *Ann. Bot* .**52**: 101-104.

Brunel, N., Leduc, N., Poupard, P., Simoneau, P., Mauget, J. C. and Viemont, J. D. (2002). KNAP2, a class I KN1-like gene is a negative marker of bud growth potential in apple trees (*Malus domestica* [L.] Borkh.). *J. Exp. Bot.* **53**: 2143–2149.

Bunn, E., Dixon, K. W. and Langley, M. A. (1989). *In vitro* propagation of *Leucopogon obtectus* Benth. Epacridaceae. *Plant Cell. Tiss. Org. Cult.***19**: 77-84.

Bunn, E. a. D., K.W. (1996*a*). Chapter 5 Tissue culture of rare and endangered Australian plants. A. M. Taji and R. R. Williams. *Tissue Culture of Australian Plants: past, present and future.* Armidale, Australia, University of New England Press**:** 157-179.

Burke, J. F., Ed. (1996). *PCR Essential Techniques*. UK, John Wiley & Sons.

Buss, L. W. (1982). Somatic cell parasitism and the evolution of somatic tissue compatibility. *Proc. Natl. Acad. Sci. U.S.A.* **79**: 5337-5341.

Caboni, E., Lauri, P., Tonelli, M., Falasca, G. and Damiano, C. (1996). Root induction by *Agrobacterium rhizogenes* in walnut. *Plant Sci.* **118**: 203-208.

Caetano-Anolles, G., Bassam, B. J. and Gresshoff, P. M. (1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* **9**: 553-557.

Caetano-Anolles, G., Bassam, B. J. and Gresshoff, P. M. (1992). DNA fingerprinting - MAAPing out a RAPD redefinition. *Bio/Technology* **10**: 937.

Cahalan, C. M. and Jinks, R. L. (1992). Vegetative propagation of Clones of Ash (*Fraxinus excelsior* L.), Sycamore (*Acer pseudoplatanus* L.) and Sweet Chestnut (*Castanea sativa* Mill.) in Britain. *Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species.*, Bordeaux, AFOCEL.

Campbell, M. A., Suttle , J. C. and Sell, T. W. (1996). Changes in cell cycle status and expression of $p34^{cdc2}$ kinase during potato tuber meristem dormancy. *Physiol. Plant.* **98**: 743–752.

Campbell, M. M., Brunner, A. M., Jones, H. M. and Strauss, S. H. (2003). Forestry's fertile crescent: the application of biotechnology to forest trees. *Plant Biotech. J.* **1**: 141-154.

Campen, R., Weston, G., Howard, B. and Harrison-Murray, R. (1990). Enhanced rooting potential in MM106 apple rootstock shoots grown in a polythene tunnel. *J. Hort. Sci.* **65**: 367-374.

Capellades, M., Fontarnau, R., Carulla, C. and Debergh, P. (1990). Environment inflences anatomy of stomata and epidermal cells in tissue cultured *Rosa multiflora*. *J. Am. Soc. Hort. Sci.* **115**: 141-145.

Capossela, A., Silander Jr J. A., Jansen, R. K., Bergen, B. and Talbot, D. R. (1992). Nuclear ribosomal DNA variation among ramets and genets of white clover. *Evolution* **46**: 1240-1247.

Carolan, J. C., Hook , I. L. I., Walsh J.J. and Hodkinson, T. R. (2002). Using aflp markers for species differentiation and assessment of genetic variability of *in vitro*-cultured *Papaver bracteatum* (section *Oxytona*). *In Vitro Cell. Dev. Biol. - Plant* **38**: 300-307.

Carvalho, L. C., Goulao, L., Oliveira, C., Goncalves, J. C. and Amancio, S. (2004). RAPD assessment for identification of clonal identity and genetic stability of in vitro propagated chestnut hybrids. *Plant Cell. Tiss. Org. Cult.* **77**: 23-27.

Castelfranco, P. and Beale, S. (1983.). Chlorophyll biosynthesis: recent advances and areas of current interest. *Ann. Rev. Plant Physiol.* **34**: 241-278.

Chakraborty, R. and Danker-Hopfe, H. (1991). Analysis of population structure: A comparative study of different estimators of Wright's fixation indices. C. R. Rao and R. Chakraborty. *Statistical Methods in Biological and Medical Sciences.*, Elsevier Science Publishers.

Chalupa, V. (1983). Micropropagation of conifer and broadleaved forest trees. *Commun. Inst. For. Czech.* **13**: 7-39.

Chalupa, V. (1984). *In vitro* propagation of oak (*Quercus robur* L.) and linded (*Tilia cordata* Mill.). *Biol. Plant* **26**: 374-377.

Chalupa, V. (1987). Effect of benzylaminopurine and thidiazuron on in vitro shoot proliferation of *Tilia cordata* Mill., *Sorbus aucuparia* L. and *Robinia pseudoacacia* L. *Biol. Plant (Praha)* **29**: 425-429.

Chalupa, V. (1987). European Hardwoods. J. M. Bonga and D. J. Durzan. *Cell and Tissue Culture in Forestry*. Dorecht, Martinus Nijhoff. **3:** 224 - 246.

Chalupa, V. (1987). Vegetative propagation of broadleaved woody species by cuttings and by an *in vitro* method. *Lesnictvi* **33**: 501 - 510.

Chalupa, V. (1988). Large scale micropropagation of *Quercus robur* L. using adenine-type cytokinins and thidiazuron to stimulate shoot proliferation. *Biol. Plant (Praha)* **30**: 414-421.

Chalupa, V. (1990). Micropropagation of Hornbeam (*Carpinus betulus* L.) and Ash (*Fraxinus excelsior* L.). *Biol. Plant.* **32**(5): 332-338.

Chalupa, V. (1993). Vegetative propagation of oak (*Quercus robur* and *Q. petraea*) by cutting and tissue culture. *Ann. Sci. For.* **50**: 295-307.

Chalupa, V. (1996). *Fagus sylvatica* L. (European Beech). Y. P. S. Bajaj. *Biotechnology in Agriculture and Forestry. Trees 4.* Berlin, Springer-Verlag. **35:** 138-154.

Chambers, G. K. and MacAvoy, E. S. (2000). Microsatellites: consencus and controversy. *Comp. Biochem. Physiol. B.* **126**: 455-476.

Champagnat, P., Payan, E., Champagnat, M., Barnola, P., Lavarenne, S. and Bertholon, C. (1986). La croissance rythmique de jeunes chenes pédonculés cultivés en conditions controlés uniforms. *Naturalia Monspeliensia, special issue, Colloque international sue l'arbre*: 303-338.

Chandler, W., Brown, D., Kimball, M., Philp, G., Tufts, W. and Weldon, G. (1937). Chilling requirements for opening of buds on deciduous orchard trees and some other plants in California. *Bull. 611. Calif. Agri. Expt. Sta. Berkeley*.

Chapman, H. M., Dipal, P. and Oraguzie, N. (2000). Genetic structure and colonizing success of a clonal, weedy species, *Pilosella officinarum* (Asteraceae). *Heredity* **84**: 401-409.

Chaput, M.-H., Sihachakr, D., Ducreux, G., Marie, D. and Barghi, N. (1990). Somatic hybrid plants produced by electrofusion between dihaploid potatoes: BF15 (HI), Aminca (H6) and Cardinal (H3). *Plant Cell Rep.* **9.**: 411-414.

Chase, M. W. and Hills, H. H. (1991). Silica gel: An ideal material for field preservation of leaf samples for DNA studies. *Taxon* **40**: 215-220.

Chibbar, R., Gurumurti, K. and Nanda, K. (1979). Changes in IAA oxidase activity in rooting hypocotyl cuttings of *Phaseolus mungo* L. *Experientia* **35**: 202-203.

Chilton, M., Tepfer, D., Petit, A., David, C., Casse-Delbart, F. and Tempe, J. (1982). *Agrobacterium rhizogenes* inserts T-DNA into the genome of the host plant root cells. *Nature* **295**: 432-434.

Chlyah, A. and Tran Thanh Van, M. (1975). Differential reacitivity in epidermal cells of *Begonia rex* excised and grown *in vitro*. *Physiol. Plant.* **35**: 16-20.

Chouard, P. (1951). Dormance et inhibitions des graines et des bourgeons: preparation au forcage. Thermoperiodisme. Conservatoire National des Arts et Metiers. Paris, Centre du Documentation Universitaire.

Cipriani, G., Marrazzo, M. T., Gaspero, G. d. and Testolin, R. (2001). DNA microsatellite in fruit crops: isolation, length polymorphism, inheritance, somatic stability and cross-species conservation. *Acta Hort.* **546**: 145-150.

Clark, S. E., Jacobsen, S. E., Levin, J. Z. and Meyerowitz, E. M. (1996). The *Clavata* and *Shoot Meristemless* loci competitively regulate meristem activity in *Arabidopsis*. *Development* **122**(5): 1567–1575.

Cline, M. G. (1991). Apical dominance. *Bot. Rev.* **57**: 318–358.

Cline, M. G. (1994). The role of hormones in apical dominance: new approaches to an old problem in plant development. *Physiol. Plant.* **90**(1): 230-237.

Cloutier, S. and Landry, B. S. (1994). Molecular markers applied to plant tissue culture. *In vitro Cell Dev. Biol. Plant* **30**: 32-39.

Cockerham, C. C. (1969). Variance of gene frequencies. *Evolution.* **23**: 72-84.

Cockerham, C. C. (1973). Analysis of gene frequencies. *Genetics.* **74**: 679-700.

Cockerham, C. C. and Weir, B. S. (1993). Estimation of gene-flow from F-statistics. *Evolution* **47**: 855-863.

Coen, D. M. (1997). The Polymerase Chain Reaction. F. Ausubel, R. Brent, R. Kingstonet al. *Short Protocols in Molecular Biology.* New York, John Wiley & Sons.

Coleman, W. K., Li, X.-Q., Tremblay-Deveau, E. and Coleman, S. (2003). Chemical maturation and storage performance of eleven Russet Burbank clones. *Can. J. Plant Sci.* **83**: 893-902.

Collet, G., Nowbuth, L. and Le, C. (1994). Comparison of the easy to root Jork 9 and Cepiland and the difficult to root EMLA 9 and Lancep *Malus* M9 rootstocks *in vitro*. *Adv. Hort. Sci.* **8**: 45-48.

Collins, M. T. and Dixon, K. W. (1992). Micropropagation of an Australian terrestrial orchid, *Diuris longifolia* R.Br. *Aus. J. Exp. Agric.* **32**: 262-262.

Colón-Carmona, A., Chen, D. L., Yeh, K. C. and Abel, S. (2000). Aux/IAA Proteins Are Phosphorylated by Phytochrome *in Vitro*. *Plant Physiol* **124**: 1728-1738.

Cottignies, A. (1983). Teneur en eau et dormance dans le bourgeon de Frene. *Z. Pflanzenphysiol.* **111**: 133-139.

Cottignies, A. (1986). The hydroloysis of starch as related to the interruption of dormancy in the ash bud. *J. Plant Physiol.***123**: 381-388.

Cottignies, A. (1986). The hydrolysis of starch as related to the interuption of dormancy in the ash bud. *J. Plant Physiol.***123**: 381-388.

Cottignies, A. (1990). Potentiel osmotique et potentiel hydrique du bourgeon terminal de Frene, au cours du cycle annuel. *Compt. Rend. Acad. Sci. Paris* **310:** 211-216.

Council, H. (1999). Policies and Priorities for the National Heritage. Forestry and the National Heritage. Kilkenny, Heritage Council.

Crabbé, J. and Barnola, P. (1996). Approach to bud dormancy in woody plants. G. A. Lang. *Plant Dormancy: Physiology, Biochemistry and Molecular Biology.* Wallingford, CAB International**:** 83-113.

Crozier, R. H. e. a. (1999). Mutability of microsatellites developed for the ant *Camponotus consobrinus*. *Mol. Ecol.* **8**: 271-276.

Curir, P., Van Sumere, C., Termini, A., Barthe, P., Marchesini, A. and Dolci, M. (1990). Flavonoid accumulation is corrected with adventitious roots formation in *Eucalyptus gunnii* Hook. micropropagated through axillary bud stimulation. *Plant Physiol.* **92**: 1148-1153.

Da Rocha Correa, L. and Fett-Neto, A. (2004). Effects of temperature on adventitious root development in microcuttings of *Eucalyptus saligna* Smith and *Eucalyptus globules* Labill. *J. Therm. Biol.* **29**: 315-324.

Damiano, C., Archilletti, T., Caboni, E., Lauri, P., Falasca, G., Mariotti, D. and Ferraiolo, G. (1995). *Agrobacterium* mediated transformation of almond: *in vitro* rooting through localized infection of *A. rhizogenes* w.t. *Acta Hort.* **392**: 161-169.

Damiano, C. and Monticelli, S. (1998). *In vitro* fruit trees rooting by *Agrobacterium rhizogenes* wild type infection. *Electr. J. Biotech.***1**: 189-195.

Davies, F. (1984). Shoot RNA, cambial activity and indole-3-butyric acid effectivity in seasonal rooting of juvenile and mature *Ficus pumila* cuttings. *Physiol. Plant.* **62**: 571-575.

Davies, P. J., Rastogi, R. and Law, D. M. (1991). Polyamines and their metabolism in ripening tomato fruit. H. E. Flores, R. N. Arteca and J. C. Shannon. *Polyamines and Ethylene. Biochemistry, Physiology and Interactions.*, American Society of Plant Physiologists.**:** 112-125.

De Capite, L. (1952) Combined action of p-aminophenylaulphonamide and p-amino-benzoic acid on the vascular parenchyma of Jerusalem artichoke cultured *in vitro*. *Compt. Rend. Soc. Biol.* **146**: 863-865.

de Castro, R. D., Zheng, X., Bergervoet , J. H. W., de Vos, C. H. R. and Bino, R. J. (1995). *beta*-tubulin accumulation and DNA replication in imbibing tomato seeds. *Plant Physiol.* **109**: 499-504.

de Fay, E., Vacher, V. and Humbert, F. (2000). Water-related phenomena in winter buds and twigs of Picea abies L. (Karst.) until bud-burst: A biological, histological and NMR study. *Ann. Bot.* **86**: 1097-1107.

De Fossard, R. A., Myint, A. and Lee, E. C. M. (1974). A broad spectrum tissue culture experiment with tobacco (*Nicotiana tabacum*) pith tissue culture. *Physiol. Plant.* **30**: 125-130.

De Klerk, G., Brugge, T. J., Smulders, R. and Benschop, M. (1990). Basic peroxidases and rooting in microcuttings of *Malus*. *Acta Hort.* **280**: 29-36.

De Klerk, G.-J., Brugge, J. T. and Marinova, S. (1997.). Effectiveness of indole acetic acid, indole butyric acid and naphthalene- acetic acid during adventitious root formation *in vitro* in *Malus* 'Jork 9'. *Plant Cell. Tiss. Org. Cult.* **49**: 9-44.

De Paiva Neto, B. and Otoni, W. C. (2003). Carbon sources and their osmotic potential in plant tissue culture: does it matter? *Scientia Hort.* **97**: 193-202.

Debergh, P. C. and Maene, L. J. (1981). A scheme for commercial propagation of ornamental plants by tissue culture. *Scientia Hort.* **14**: 335 - 345.

Debergh, P. C. and Read, P. E. (1991). Micropropagation. P. C. Debergh and R. H. Zimmerman. *Micropropagation: Technology and Application.* Netherlands, Kluwer Academic Publishers: 311 - 336.

DeBoer, S. and Van Elk, B. (1983). Het stekken van boomkwekwrijkewassen. Boskoop, Proefstatition voor de Boomkwekerij.

Del Duca, S. and Serafini-Fracassini, D. (1993). Polyamines and protein modification during the cell cycle. J. Ormrod and D. Francis. *Molecular and Cell Biology of the Plant Cell Cycle.* Dortrecht, Netherlands., Kluwer Academic Publishers: 143-156.

den Boer, B. G. W. and Murray, J. A. H. (2000). Control of plant growth and development through manipulation of cell-cycle genes. *Curr. Op. Biotech.* **11**: 138–145.

Devarumath, R. M., Nandy, S., Rani, V., Marimuthu, S., Muraleedharan, N. and Raina, S. N. (2002). RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. *assamica* (Assam-India type). *Plant Cell Rep.* **21**: 166-173.

Deverno, L. L., Charest, P. J. and Bonen, L. (1994). Mitochondrial DNA variation in somatic embryogenic cultures of *Larix*. *Theor. Appl. Genet.* **88**: 727-732.

Devitt, M. L. and Stafstrom, J. P. (1995). Cell cycle regulation during growth-dormancy cycles in pea axillary buds. *Plant Mol. Biol.* **29**: 255-265.

Diaz-Sala, C., Hutchison, K., Goldfarb, B. and Greenwood, M. (1996). Maturation-related loss in rooting competence by loblolly pine stem cuttings: The role of auxin transport, metabolism and tissue sensitivity. *Physiol. Plant.* **97**: 481-490.

Dieringer, D. and Schlötterer, C. (2003). Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Mol. Ecol. Notes* doi:10.1046/j.1471-8278.2003.00351.x.

Dodds, J. H. and Roberts, L. W. (1995). Experiments in Plant Tissue Culture, Cambridge University Press.

Donnelly, D., Vidaver, W. and Lee, K. (1985). The anatomy of tissue cultured red raspberry prior to and after transfer to soil. *Plant Cell. Tiss. Org. Cult.* **4**: 43-50.

Doré, J. (1965). Physiology of regeneration in cormophytes. W. Ruhland. *Encyclodedia of Plant Phsiology*. Berlin, Springer-Verlag. XV/2: 1-91.

Dowling, T. E., Moritz, C., Palmer, J. D. and Rieseberg, L. H. (1996). Nucleic Acids III: Analysis of Fragments and Restriction Sites. D. M. Hillis, C. Moritz and B. K. Mable. *Molecular Systematics*. USA, Sinauer Associates: 249-320.

Doyle, J. J. and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull. Bot. Soc. Am.***19**: 11 - 15.

Driver, J. and Suttle, G. (1987). Nursery handling of propagules. J. Bonga and D. Durzan. *Cell and Tissue Culture in Forestry. Vol. 2.* Dorecht., Martinus Nijhoff,: 320-335.

Driver, J. A. and Kuniyuki, A. H. (1984). *In vitro* propagation of Paradox walnut rootstock. *HortScience* **19**: 507-509.

Drolet , G., Dumbroff, E. B., Legge, R. L. and Thompson, J. E. (1986). Radical scavenging properties of polyamines. *Phytochem.* **25**: 367–271.

Druart, P., Kevers, C., Boxus, P. and Gaspar, T. (1982). *In vitro* promotion of root formation by apple shoots through darkness effect on endogenous phenols peroxidases. *Z. Pflanzenphysiol.* **5**: 429-436.

Durand-Cresswell, R., Boulay, M. and Franclet (1982). Vegetative propagation of *Eucalyptus*. J. M. Bonga and D. J. Durzan. *Tissue Culture in Forestry.* The Hague., Martinus Nijhoff: 150-181.

Duroux, L., Ermel-Fontaine, F., Breton, C., Charpentier, J., Capelli, P., Bruant, B., Label, P., Couee, I. and Jay-Allemand, C. (1998). How could we progress in the knowledge of the mechanisms that govern the adventitous root formation from aerial plant tissues? *Bull. Soc. Royale Sci. Liege* **67**: 105-115.

Durzan, D. J. (1988). Applications of cell and tissue culture in tree improvement. Ciba Foundation Symposium 137. Applications of plant cell and tissue culture. UK, John Wiley & Sons Ltd.

Ebert, A. and Taylor, H. F. (1990). Assessment of changes of 2,4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. *Plant Cell. Tiss. Org. Cult.* **32**: 223-225.

Edlin, H. L. (1970). Trees, Woods and Man. London, Collins.

Edwards, K. J., Barker, J. H. A., Daly, A., Jones, C. and Karp, A. (1996). Microsatellite libraries enriched for several microsatellite sequences in plants. *Biotechniques* **20**: 758-760.

Edwards, P. B., Wanjura, W. J. and Brown, W. V. (1990.). Mosaic resistance in plants. *Nature* **347**: 434.

Eeuwens, C. J. (1976). Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro*. *Physiol. Plant.* **36**: 23-28.

Einset, J. W. and Alexander, J. H. (1985). Multiplication of *Syringa* species and cultivars in tissue culture. *Comb. Proc. Int. Plant. Prop. Soc.* **3**: 628-636.

El Mousadik, A. and Petit, R. J. (1996). High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. T. *Theor. Appl. Genet.* **92**: 832-839.

Eliasson, L. (1972). Translocation of shoot applied indolylacetic acid into the roots of *Populus tremula*. *Physiol. Plant.* **27**: 412-416.

Eliasson, L. and Areblad, K. (1984). Auxin effect on rooting in pea cuttings. *Physiol. Plant.* **61**: 293-297.

Ellegren, H. (2000a). Microsatellite mutations in the germline: implications for evolutionary inference. *Trends Genet.* **16**: 551- 558.

Ellegren, H. (2000b). Heterogeneous mutation processes in human microsatellite DNA sequences. *Nat. Genet.* **24**: 400-402.

Elsik, C. G., Minihan, V. T., Hall, S. E., Scarpa, A. M. and Williams, C. G. (2000). Low-copy microsatellite markers for *Pinus taeda* L. *Genome* **43**: 550-555.

Erez, A. (2000). Bud dormancy: a suggestion for its control mechanism and its evolution. J. D. Viemont and J. Crabbe. *Dormancy in plants.*, CAB International.

Erez, A., Couvillon, G. A. and Kays, S. J. (1980). The effect of oxygen concentration on the release of Peach leaf buds from rest. *HortScience* **15**: 39 - 41.

Erez, A., Faust, M. and Line, M. J. (1998). Changes in water status in peach buds in induction, developement and release from dormancy. *Scientia Hort.* **73**: 111-123.

Erez, A., Wang, S. Y. and Faust, M. (1997). Lipids in peach buds during dormancy, a possible involvement in dormancy control. *Adv. Hort. Sci.* **11**: 128-132.

Erez, A. and Yablowitz, Z. (1981). Rooting of peach hardwood cuttings for the meadow orchard. *Scientia Hort.* **15**: 137-144.

Eriksen, E. (1974). Root formation in pea cuttings. III. The influence of cytokinin at different developmental stages. *Physiol. Plant.* **30**: 163-167.

Escobar-Gutierrez, A. J. and Gaudilltre, J. P. (1996). Distribution, metabolisme et role du sorbitol chez les plantes suptrieures. *Agronomie* **16**: 281-298.

Essiamah, S. and Eschrich, W. (1986). Water uptake in deciduous trees during winter and the role of conducting tissues in spring reactivation. *IAWA Bulletin n. s.* **7**: 31-38.

Evans, D. A., Sharp, W. R. and Medina Filho, H. P. (1984). Somaclonal and gametoclonal variation. *Am. J. Bot.* **71**: 759-774.

Evans, P. T. and Malmberg, R. L. (1989). Do polyamines have roles in plant development? *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **40**: 235-269.

Ewald, D. and Naujoks (1997). Sustainability of rejuvenation in Larch and Oak clones. Cost 882, 4th Meeting Working Group 3., Nitra, Slovakia.

Fabijan, D., Taylor, J. S. and Reid, D. M. (1981). Adventitious rooting in hypocotyls of sunflower (*Helianthus annuus*) seedlings. II. Action of gibberellins, cytokinins, auxins and ethylene. *Physiol. Plant.* **53**: 589-597.

Fagerström, T., Briscoe, D. A. and Sunnucks, P. (1998). Evolution of mitotic cell-lineages in multicellular organisms. *Trends Ecol. Evol.* **13**: 117-120.

Fahleson, J., Dixelius, J., Sundberg, E. and Glimelius, K. (1988). Correlation between flow cytometric determination of nuclear DNA content and chromosome number in somatic hybrids within *Brassicaceae*. *Plant Cell Rep.* **7**: 74-77.

Falkinier, F. R. (1988). Strategy for the selection of antibiotics for use against common bacterial pathogens and endophytes of plants. *Acta Hort.* **225**: 53-56.

Fan, H.-Y., He, X., Kingston, R. E. and Narlika, G. J. (2003). Distinct strategies to make nucleosomal DNA accessible. *Molecular Cell* **11**(5): 1311–1322.

Fasolo, F., Zimmerman, R. H. and Fordham, I. (1989). Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivar. *Plant Cell. Tiss. Org. Cult.* **16**: 75-87.

Faust, M., Erez, A., Rowland, L., Wang, S. and Norman, H. (1997). Bud dormancy in perennial fruit trees: physiological basis for dormancy induction, maintenance, and release. *HortScience* **32**: 623-629.

Faust, M., Liu, D., Line, M. J. and Stutte, G. W. (1995). Conversion of bound to free water in endodormant buds of apple is an incremental process. *Acta Hort.* **395**: 113-118.

Faust, M., Liu, D., Merle, M. and Stutte, G. W. (1991). Bound versus free water in dormany apple buds - a theory for endodormancy. *HortScience* **26**: 887-890.

Faust, M. and Wang, S. Y. (1992). Polyamines in horticulturally important plants. *Plant Breeding Rev.* **14**: 333-356.

Favre, J. M. and Juncker, B. (1987). *In vitro* growth of buds taken from seedlings and adult plant material in *Quercus robur* L. *Plant Cell. Tiss. Org. Cult.* **8**: 49-60.

Fedoroff, N. (2002). Cross-talk in abscisic acid signaling. *Science STKE Review* **10**.

Ferguson, I. B., Lurie, S. and Bowen, J. H. (1994). Protein synthesis and breakdown during heat shock of cultured pear cells. *Plant Physiol.* **92**: 1429 - 1437.

Fett-Neto, A. G., Fett, J. P., Goulart, L. W. V., Pasquali, G., Termignoni, R. R. and Ferreira, A. G. (2001.). Distinct effects of auxin and light on adventitious root development in *Eucalyptus saligna* and *Eucalyptus globulus*. *Tree Physiol.* **21**: 457-464.

Feuser, S., Meler, K., Daquinta, M., Guerra, M. P. and Nodari, R. O. (2003). Genotypic fidelity of micropropagated pineapple (*Ananas comosus*) plantlets assessed by isozyme and RAPD markers. *Plant Cell. Tiss. Org. Cult.* **72**: 221-227.

Fink, S. (1972). Adventitious root primordia: The cause of abnormally broad xylem rays in hard- and softwoods. *International Association of Wood Anatomists.* **3**: 31-38.

Finlayson, S. A., Lee, I.-J. and Morgan, P. W. (1998). Phytochrome b and the regulation of circadian ethylene production in sorghum. *Plant Physiol.***116**: 17-25.

Fiola, J. A., Hassan, M. A., Swartz, H. J., Bors, R. H. and McNicols, R. (1990). Effect of thidiazuron, light fluence rates and kanamycin on *in vitro* shoot organogensis from excised Rubus cotyledons and leaves. *Plant Cell. Tiss. Org. Cult.* **20**: 223-228.

Fisher, P. J., Gardner, R. C. and Richardson, T. E. (1996). Single locus microsatellites isolated using 5' anchored PCR. *Nucleic Acid Res.* **24**: 4369-4371.

Flores, H. and Protacio, C. (1990). polyamine metabolism in plant cell and organ culture. H. E. Flores. *polyamines and Ethylene: Biosynthesis, Physiology and Interactions.*, Amer. Soc. Plant Physiologists.

Flores, H. E. (1991). Changes in polyamine metabolism in response to abiotic stress. R. D. Slocum and H. E. Flores. *Biochemistry and Physiology of Polyamines in Plants.* Boca Raton, Florida, CRC Press Inc.: pp 214-225.

Flores, H. E. a. M.-T., J. (1991). Polyamines and plant secondary metabolites. R. D. Slocum, Flores, H. E. *Biochemistry and Physiology of Polyamines in Plants.* Boca Raton, Florida, CRC Press Inc.: pp 57-76.

Ford-Lloyd, B. and Jackson, M. (1986). Plant Genetic Resources: An Introduction to their Conservation and Use. London, Edward Arnold.

Fossard, D. (1977). The Horizons of Tissue Culture Propagation Seminar NSW ASS., Nurserymen University, Sydney.

Fossati, T., Grassi, F., Sala, F. and Castiglione, S. (2003). Molecular analysis of natural populations of *Populus nigra* L. intermingled with cultivated hybrids. *Mol. Ecol.* **12**: 2033-2043.

Fourre, J. L., Berger, P., Niquet, L. and Andre, P. (1997). Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. *Theor. Appl. Genet.* **94**: 159-169.

Foyer, C., Rowell, J. and Walker, D. (1983). Measurement of the ascorbate content of spinach leaf protoplasts during illumination. *Planta* **157**: 239 - 244.

Franclet, A. (1983). Rajuenissement, culture *in vitro* et pratique sylvicloe. *B. Soc. Bot. Fr.* **2**: 87.

Franclet, A., Boulay, M., Bekkaoui, F., Fouret, Y., Verschoore-Martouzet, B. and Walker, N. (1987). Rejuvenation. J. M. Bonga and D. J. Durzan. *Cell and Tissue Culture in Forestry*. Dordrect, Martinus Nijhoff. **1:** 232 - 248.

Freeman, D., Riou-Khamlichi, C., Oakenfull, E. A. and Murray, J. A. H. (2003). Isolation, characterization and expression of cyclin and cyclin-dependent kinase genes in Jerusalem artichoke (*Helianthus tuberosus* L.). *J. Exp. Bot.* **54**(381): 303–308.

Frewen, B. E., Chen, T. H. H., Howe, G. T., Davis, J., Rohde, A., Boerjan, W. and Bradshaw, H. D. (2000). Quantitative trait loci and candidate gene mapping of bud set and bud flush in *Populus*. *Genetics* **154**: 837–845.

Fridborg, G. and Eriksson, T. (1975). Effects of activated charcoal on growth and morphogenesis in cell cultures. *Physiol. Plant.* **34**: 306-308.

Fridborg, G., Pedersen, M., Landstrom, L. E. and Eriksson, T. (1978). The effects of activated charcoal on tissue culture: absorption of metabolites inhibiting morphogenesis. *Physiol. Plant.* **43**: 104-106.

Fries, N. (1960). The effect of adenine and kinetin on growth and differentiation of *Lupinus*. *Physiol. Plant.* **13**: 468-481.

Fuchigami, L. H. and Nee, C. C. (1987). Degree growth stage model and rest breaking mechanisms in temperate woody perennials. *HortScience* **22**: 836--845.

Gagneux, P., Boesch, C. and Woodruff, D. S. (1997). Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hairs. *Mol. Ecol.* **6**: 861-868.

Galston, A. W. (1983). Polyamines as modulators of plant development. *BioScience* **33**: 382-388.

Galston, A. W. and Kaur-Sawhney, R. (1995). Polyamines as endogenous growth regulators. P. J. Davies. *Plant Hormones: Physiology, Biochemistry and Molecular Biology.* Dordrecht, Kluwer Academic Publishers: 158-178.

Galston, A. W. and Kuar-Sawhney, Y. R. (1987). Polyamines as endogenous growth regulators. P. J. Davies. *Plant Hormones and Their Role in PLant Growth and Development.* Dordrecht, Netherlands, Martinus Nijhoff: 280-295.

Galston, A. W. and Kuar-Sawhney, Y. R. (1990). Polyamines in plant physiology. *Plant Physiol.* **94**: 406-410.

Galston, A. W., Sawhney, R. K., Altman, A. and Flores, H. (1980). Polyamines, macromolecular syntheses and the problem of cereal protoplast regeneration. L. Ferenczy and G. L. Farkas. *Protoplast Research.* Oxford, Pergamin Press: 485-497.

Gamborg, O., Constabel, F. and Shyluk, J. (1974). Organogenesis in callus from shoot apices of *Pisum sativum*. *Physiol. Plant.* **30**: 125-128.

Gamborg, O. L., Miller, R. A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean roots cells. *Exp. Cell Res.* **50**: 151-158.

García-Gómez, M., Sánchez-Romero, C., Barceló-Munoz, A., Heredia, A. and Pliego-Alfaro, F. (1994). Levels of endogenous indole-3-acetic acid and indole-3-acetylaspartic acid during adventitious rooting in avocado microcuttings. *J. Exp. Bot.* **45**: 865-870.

Garelkova, Z., Izvorska, N. and Marinov, I. (1991). The influence of hormone medium upon the rapid regeneration *in vitro* explants of *Fraxinus excelsior* L. *Compt. Rend. l'Acad. Bulgare Sci.* **44**(10): 105-108.

Garrido, G., Guerrero, J. R., Cano, E. A., Acosta, M. and Sànchez-Bravo, J. (2002). Origin and basipetal transport of the IAA responsible for rooting of carnation cuttings. *Physiol. Plant.***114**: 303-312.

Gaspar, T. and Hausman, J. (2002). Auxins in the biology of roots. Y. Waisel, Eshel, A., Kafkafi, U. *Plant Roots: The Hidden Half.* New York., Marcel Dekker Inc.: 383-403.

Gautheret, R. J. (1945). Une voie nouvelle en biologie végétale: la culture des tissus. Paris, Gallimard.

Gautheret, R. J. (1985). History of plant tissue and cell culture: A personal account. I. K. Vasil. *Cell Culture and Somatic Cell Genetics*. New York, Academic Press. **2:** 1 - 59.

Gawal, N. J. and Jarret, R. L. (1991). A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea. Plant Mol. Biol. Rep.* **9**: 262-266.

Geneve, R., Hackett, W. and Swanson, B. (1988). Adventitious root initiation in de-bladed petioles from the juvenile and mature phases of English ivy. *J. Amer. Soc. Hort. Sci. s* **113**: 630-635.

Geneve, R. L., Kester, S. T. and Yusnits, S. (1992). Micropropagation of Eastern Redbud (*Cercis Canadensis* L.). *Int. Plant. Prop. Soc.* **42**: 417-420.

George, E. F. and Sherrington, P. D. (1984). Plant propagation by tissue culture. Basingstoke, Exegetics.

George, G. F. (1993). Plant Propagation by Tissue Culture Part 1: The Technology. Edington, Wilts, Exegetics Limited.

George, G. F. (1996). Plant Propagation by Tissue Culture Part 2: The Technology. Edington, Wilts, Exegetics Limited.

Gill, D. E. (1986). Individual plants as genetic mosaics: ecological organisms versus evolutionary individuals. M. J. Crawley. *Plant Ecology*. Oxford, Blackwell**:** pp. 321-343.

Gill, D. E., Chao, L., Perkins, S. L. and Wolf, J. B. (1995). Genetic mosaicism in plants and clonal animals. *Annu. Rev. Ecol. Syst.* **26**: 423-444.

Gill, D. E. and Halversson, T. G. (1984). Fitness variations among branches within trees. B. Shorrocks. *Evolutionary Ecology*. Oxford, Blackwell Scientific Publications**:** 105-116.

Gilmour, S. J. and Thomashow, M. F. (1991). Cold acclimation and coldregulated gene expression in ABA mutants of *Arabidopsis thaliana*. *Plant Mol. Biol.* **16**: 1233–1240.

Girouard, R. (1967). Initiation and development of adventitious roots in stem cuttings of *Hedera helix*. *Canadian Journal of Botany*. **45**: 1883-1886.

Glenn, T. C. e. a. (1996). Allelic diversity in alligator microsatellite loci is negatively correlated with GC content of flanking sequences and evolutionary conservation of PCR amplifiability. *Mol. Biol. Evol.* **13**: 1151-1154.

Goffreda, J. C., Szymkowiak, E. J., Sussex, I. M. and Mutschler, M. A. (1990). Chimeric tomato plants show that aphid resistance and triacylglucose production are epidermal autonomous characters. *The Plant Cell* **2**: 643-649.

Goldstein, D. B. and Pollock, D. D. (1997). Launching microsatellites: a review of mutation processes and methods of phylogenetic interference. *J. Hered.* **88**: 335-342.

Gonzalez, A. I., Pelaez, M. I. and Ruiz, M. L. (1996). Cytogenetic variation in somatic tissue cultures and regenerated plants of barley (*Hordeum vulgare* L.). *Euphytica* **91**: 37-43.

Goossens, B., Waits, L. P. and Taberlet, P. (1998.). Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. *Mol. Ecol.* **7**: 1237-1241.

Goto, S., Thakur, R. C. and Ishii, K. (1998). Determination of genetic stability in long-term micropropagated shoots of *Pinus thunbergii* Parl. using RAPD markers. *Plant Cell Rep.* **18**: 193-197.

Goudet, J. (1995). FSTAT (version 1.2): a computer programme to calculate F-statistics. *Journal of Heredity* **86**: 485-486.

Greenwood, M., Diaz-Sala, C., Singer, P., Decker, A. and Hutichison, K. (1997). Differential gene experssion during maturation-caused decline in adventitious rooting ability in lobolly pine (*Pinus taeda* L.). A. Altman and Y. Waisel. *Biology of root formation and development.* New York, Plenum Press: 203-208.

Greenwood, M. S. (1995). Juvenility and maturation in conifers: current concepts. *Tree Physiol.* **15**: 433-438.

Greenwood, M. S. and Hutchison, K. W. (1993). Maturation as a development process. M. R. Ahuja and W. J. Libby. *Clonal Forestry*. Berlin, Springer-Verlag. **1:** 14-33.

Grieve, M. (1981). A Modern herbal. New York, Dover Publications, Inc.

Grigoriadou, K., Vasilakakis, M. and Eleftheriou, E. P. (2002). *In vitro* propagation of the Greek olive cultiva 'Chondrolia Chalkidikis'. *Plant Cell. Tiss. Org. Cult.* **71**: 47-54.

Grosberg, R. K. and Strathmann, R. R. (1998). One cell, two cell, red cell, blue cell: the persistence of a unicellular stage in multicellular life histories. *Trends Ecol. Evol.* **13**: 112-116.

Gupta, P., Mascarenhas, A. and Jagannathan, V. (1981). Tissue culture of forest trees: clonal propagation of mature trees of *Eucalyptus citriodora* by tissue culture. *Plant Sci. Lett.* **20**: 195-201.

Gupta, P. K., Metha, U. and Mascarenhas, A. F. (1983). A tissue culture method for rapid multiplication of mature trees of *Eucalyptus torelliana* and *E. camaldulensis*. *Plant Cell Rep.* **2**: 296.

Gupta, P. K., Timmis, R. and Mascarenhas, A. F. (1991). Field performance of micropropagated forestry species. *In Vitro Cell. Dev. Biol.* **27**: 159 -164.

Gurley, W. B. and Key, J. L. (1991). Transcriptional regulation of the heat shock response: a plant perspective. *Biochemistry* **30**: 1 - 12.

Gutierrez, C., Ramirez-Parra, E., Castellano, M. M. and del Pozo , J. C. (2002). G1 to S transition: more than a cell cycle engine switch. *Curr. Opin. Plant Biol.* **5**: 480–486.

Gyulai, G., Janovszky, J., Kiss, E., Lelik, L., Csillag, A. and Heszky, L. E. (1992). Callus initiation and plant regeneration from inflorescence primordial of the intergeneric hybrid *Agropyron repens* (L.) Beauv. X *Bromus inermis* Leyss. cv. Nanus on a modified nutritive medium. *Plant Cell Report* **11**: 266-269.

Haberlandt, G. (1902). Kulturversuche mit isolierten Pflazenzellen. *Sitzungsber. Acad. Wiss. Wein. Math. Nat. Classe III* **1**: 69 - 92.

Hackett, W. (1985). Juvenility, maturation, and rejuvenation in Woody plants. J. Janick. *Horticultural Reviews*. Westport, Connecticut, AVI Publishing Company. **7**: 109-156.

Haissig, B. (1970). Influence of indole-3-acetic acid on adventitious root primordial of brittle willow. *Planta* **95**: 27-35.

Haissig, B. (1974). Origins of adventitious roots. *New Zeal. J. For. Sci.* **4**: 299-310.

Hallé, F., Oldeman, R. A. A. and Tomlinson, P. B. (1978). Tropical trees and forests: An architectural analysis. Berlin, Springer-Verlag.

Hamant, O., Hamant, O., Nogue, F., Belles-Boix , E., Jublot, D., Grandjean, O., Traas, J. and Pautot, V. (2002). The KNAT2 homeodomain protein interacts with ethylene and cytokinin signaling. *Plant Physiol.* **130**: 657-665.

Hammatt, N. (1994). Shoot initiation in the leaflet axils of compound leaves from micropropagated shoots of juvenile and mature common ash (*Fraxinus excelsior* L.). *J. Exp. Bot.* **45**(275): 871 - 875.

Hammatt, N. (1997). *Fraxinus excelsior* L. (Common Ash). *Biotechnol. Agric. For.* **35**: 173 - 193.

Hammatt, N. and Grant, N. J. (1993). Apparent rejuvenation of wild cherry (*Prunus avium* L.) during micropropagation. *J. Plant Physiol.* **141**: 341 - 346.

Hammatt, N. and Ridout, M. S. (1992). Micropropagation of common ash (*Fraxinus excelsior*). *Plant Cell. Tiss. Org. Cult.* **13**: 67 - 74.

Hammerschlag, F. (1982). Factors affecting establishment and growth of peach shoots *in vitro*. *HortScience* **17**: 85-86.

Hammerschlag, F., Bauchan, G. and Scorza, R. (1987). Factors affecting *in vitro* multiplication and rooting of peach cultivars. *Plant Cell. Tiss. Org. Cult.* **8**: 235-242.

Han, K.-H., Shin, D.-I. and Keathley, D. E. (1997). Tissue culture responses of explants taken from branch sources with different degrees of juvenility in mature black locust (*Robinia pseudoacacia*) trees. *Tree Physiol.* **17**: 671-675.

Hansen, M., J., , Pohlhein, F. and Obermeier, C. (2004). Development of RAPD markers for studies on a *Populus*-chimera. *Acta Hort.* **651**: 69-74.

Harada, H. and Murai, Y. (1995). Cultivar differences in shoot proliferation and rooting of Japanese plum (*Prunus salicina* Lindl.). *Comb. Proc. Int. Plant. Prop. Soc.*. **45**: 627-630.

Harbourne, M. (2004). Genetic characterisation of common ash (*Fraxinus excelsior* L.) in Ireland and around Europe. Phd. Thesis (draft). University of Dublin.

Harrar, Y., Bellec, Y., Bellini, C. and Faure, J. D. (2003). Hormonal control of cell proliferation requires PASTICCINO genes. *Plant Physiol.* **132**: 1217–1227.

Harris, G. and Hart, E. (1964). Regeneration from leaf squares *of Peperomia sandersii* A, DC: a relationship between rooting and budding. *Ann. Bot.* **28**: 509-526.

Hartmann, H., Kester, D., Davies, F. (1990). Plant Propagation Principle and Practices. Prentice Hall, Englewood Cliffs.

Hartmann, H. and Kester, D. (1975). Plant Propagation: Principles and Practices. New Jersey, Prentice-Hall.

Hausman, F. (1993). Changes in peroxidase activity, auxin level and ethylene production during root formation by poplar shoots raised *in vitro*. *Plant Growth Regul.***13**: 263-268.

Hausman, J., Kevers, C. and Gaspar, T. (1994). Involvement of putrescine in the inductive rooting phase of poplar. *Physiol. Plant.* **92**: 201-206.

Hausman, J., Kevers, C. and Gaspar, T. (1995). Putrescine control of peroxidase activity in the inductive phase of rooting in poplar shoots *in vitro*, and the adversary effect of spermidine. *J. Plant Physiol.* **146**: 681-685.

Heiman, P. J. and E., P. J. (1983). Aseptic micropropagation of *Fraxinus pennsylvanica* Marsh. and *Fraxinus americana* L. utilizing shoot tip explants. *HortScience* **18**: 617.

Heloir, M., Kevers, C., Hausman, J. and Gaspar, T. (1996). Changes in the concentrations of auxins and polyamines during rooting of *in vitro* propagated walnut shoots. *Tree Physiol.* **16**: 515-519.

Heloir, M. C., Fournioux, J. C., Barbier, M., Jeandet, P. and Bessis, R. (1998). Endogenous polyamine concentrations in juvenile, adult and micropropagated grapevine (*Vitis vinifera* L. cv. Pinot noir). *Vitis* **37**: 61-62.

Henchoz, S., Chi, Y., Catarin, B., Herskowitz, I., Deshaies, R. J. and Peter, M. (1997). Phosphorylation- and ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor Far1p in budding yeast. *Genes Dev.* **11**: 3046–3060.

Henshaw, G. G. (1984). Tissue culture for disease elimination and germplasm storage. P. B. Vose and S. G. Blixt. *Crop Breeding: A Contemporary Basis*. Oxford, Pergamon Press.

Henzell, R. F., Briscoe, M. R. and Gravett, I. (1991). Improving kiwifruit vine productivity with plant growth regulators. *Acta Hort.* **297**: 345–350.

Herman, E. B. (2000). Regeneration and Micropropagation: Techniques, Systems and Media 1997-1999, Agritech Consultants.

Heuertz, M., Hausman, J., Tsvetkov, I., Frascaria-Lacostes, N. and Vekemans, X. (2001). Assessment of genetic structure within and among Bulgarian populations of the common ash (*Fraxinus excelsior* L.). *Mol. Ecol.* **10**: 1615 - 1623.

Heuertz, M., Hausman, J.-F., Hardy, O. J., Vendramin, G., Frascaria-Lacoste, N. and Vekemans, X. (2004). Nuclear microsatellites reveal contrasting patterns of genetic structure between western and southeastern european populations of the common ash (*Fraxinus excelsior* L.). *Evolution* **58**: 976-988.

Heuertz, M., Vekemans, X., Hausman, J., Palada, M. and Hardy, O. J. (2003). Estimating seed vs. pollen dispersal from spatial genetic structure in the common ash. *Mol. Ecol.* **12**: 2483-2495.

Hiatt, A. (1989). Polyamine synthesis in maize cell lines. *Plant Physiol.* **90**: 1378-1381.

Hill, A. and Campbell, G. (1949). Prolonged dormancy of deciduous fruit -trees. *Empire J. Expt. Agr.* **17**: 259-264.

Hillis, D. M., Mable, B. K., Larson, A., Davis, S. K. and Zimmer, E. A. (1996). Nucleic Acids IV: Sequencing and Cloning. D. M. Hillis, C. Moritz and B. K. Mable. *Molecular Systematics*. USA, Sinauer Associates: 321-318.

Hillman, J. R. (1984). Apical dominance. M. B. Wilkins. *Advanced plant physiology*. London, Pitman: 127-148.

Hillman, J. R. (1985). Apical Dominance. M. B. Wilkins. *Advanced Plant Physiology*. London, Pitman: 127-148.

Hirochika, H., Sugimoto, K., Tsugawa, H. and Kauda, M. (1996). Retrotransposons of rice involved in mutations induced by tissue culture. *P Natl. Acad. Sci. USA* **93**: 7783-7788.

Hocquigny, S., Pelsy, F., Dumas, V., Kindt, S., Heloir, M.-C. and Merdinoglu, D. (2004). Diversification within grapevine cultivars goes through chimeric states. *Genome* **47**: 579-589.

Hodkinson, T. R., Chase, M. W. and Renvoize, S. A. (2002). Characterisation of a Genetic Resource Collection for *Miscanthus* (Saccharinae, Andropogoneae, Poaceae) using AFLP and ISSR PCR. *Ann. Bot.* **89**: 627-636.

Horvath, D. P. and Anderson, J. V. (2000). The effect of photosynthesis on underground adventitious shoot bud dormancy/quiescence in leafy spurge (*Euphorbia esula*). J.-D. Vie´mont and J. Crabbe´. *2nd International Symposium On Plant Dormancy: Short Communications*, Presses de l'University d'Angers: 30–34.

Horvath, D. P., Anderson, J. V., Chao, W. S. and Foley, M. E. (2003). Knowing when to grow: signals regulating bud dormancy. *Trends Plant Sci.* **8**: 534-540.

Horvath, D. P., Chao, W. S. and Anderson, J. V. (2002). Molecular analysis of signals controlling dormancy and growth in underground adventitious buds of leafy spurge. *Plant Physiol.* **128**: 1439-1446.

Howard, B. (1968). The influence of 2-indole-3-butyric acid and basal temperature on the rooting of apple rootstock. *J. Hortic. Sci.* **43**: 23-31.

Howard, B. (1981). Propagation by leafless winter cuttings. *The Plantsman* **3**: 99-107.

Howard, B. and Harrison-Murray, R. (1988). Effects of water status on rooting and establishment of leafless winter (hardwood) cuttings. *Acta Hort.* **227**: 134-140.

Hu, X., and Worton, R. G. (1992). Partial gene duplication as a cause of human disease. *Hum. Mutat.* **1**: 3-12.

Humphries, E. (1960). Kinetin inhibited root formation on leaf petioles of detached leaves of *Phaseolus vulgaris* (dwarf bean). *Physiol. Plant.* **13**: 659-663.

Hurlbert, S. H. (1971). The nonconcept of species diversity: a critique and alternative parameters. *Ecology* **52**: 577-586.

Hutteman, C. A. and Preece, J. E. (1993). Thidiazuron: A potent cytokinin for woody plant tissue culture. *Plant Cell. Tiss. Org. Cult.* **33**: 105-119.

Isabel., N., Tremblay, L., Michaud, M., Tremblay, F. M. and Bousquet, J. (1993). RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill) BSP. *Theor. Appl. Genet.* **86**: 81-87.

Iwamasa, M., Nishuiura, H., Okudai, N. and Ishiughi, D. (1977). Characteristics due to chimeras and their stability in citrus cultivars. *Proc. Inter. Soc. Cit.* **2**: 571-574.

Jain, S. M. (2001). Tissue culture-derived variation in crop improvement. *Euphytica* **118**: 153-166.

Jaligot, E., Rival, A., Beule, T., Dussert, S. and Verdeil, J. L. (2000). Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. *Plant Cell Rep.* **19**: 684-690.

Jarret, R. L., Hasegawa, P. M. and Erickson, H. T. (1980). Effects of medium components on shoot formation from cultured tuber discs of potato. *J. Am. Soc. Hort. Sci.* **105**: 238 - 242.

Jarvis, B., and Booth, A. (1981). Influence of indole-3-butryic acid, boron, myo-inositol, vitamin D2 and seedling age on adventitious root development in cuttings of *Phaseolus aureus*. *Physiol. Plant.* **53**: 213-218.

Jarvis, B. (1986). Endogenous control of adventitious rooting in non-woody cuttings. M. Jackson. *New Root Formation in Plants and Cuttings.* Dordrecht, Martinus Nijhoff: 141-190.

Jay-Allemand, C., Doumas, P., Sotta, B., Tranvan, H., Miginiac, E. (1995). Juvenility and physiology of rhizogenesis in two woody species (*Sequoia sempervirens* and *Julgans nigra x Julgans regia*). H. Saudermann Jr., Bonnet-Masimbert, M. *EUROSILVA-Contribution to forest tree physiology.* Paris, INRA Editions (Les Colloques, no 76): 79-99.

Jeandroz, S., Frascaria-Lacoste, N. and Bousquet, J. (1996). Molecular recognition of the closely related *Fraxinus excelsior* and *F. oxyphylla* (Oleaceae) by RAPD markers. *For. Gen.* **3**: 237-242.

Jensen, P. J., Hangarter, R. P. and Estelle, M. (1998). Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown *Arabidopsis*. *Plant Physiol.* **116**: 975-979.

Johnson, K. A. (1996.). Chapter 2 The application of *in vitro* technology to Australian plants (with particular emphasis on the nutritional requirments). A. M. Taji and R. R. Williams. *Tissue Culture of Australian Plants: past, present and future.* Armidale, Australia, University of New England Press: 16-55.

Jona, R. and Vigliocco, R. (1985). Axillary bud culture of peach. *Acta Hort.* **173**: 223-228.

Jones, O. P. and Hadlow, W. C. C. (1989). Juvenile-like character of apple trees produced by grafting scions and rootstocks produced by micropropagation. *J. Hort. Sci* .**64**: 395 - 401.

Joustra, M. and Verhoeven, P. (1984). Rooting of hardwood cuttings of certain woody perennial species. *Plant Propagator* **30**: 3-4.

Jouve, L., Fouché, J. G. and Gaspar, T. (1995). Early biochemical changes during acclimation of Poplar to low temperature. *J. Plant Physiol.***147**: 247-250.

Juahar, P. P. (1996). Methods of genome analysis in plants. Boca Raton, CRC Press, Inc.

Kadota, M., Imizu, K. and Hirano, T. (2001). Double-phase *in vitro* culture using sorbitol increases shoot proliferation and reduses hyperhydricity in Japanese pear. *Scientia Hort.* **89**: 207-215.

Kaira, G. and Bhatla, S. (1998). Auxin - calcium interaction in adventitious root formation in the hypocotyl explants of sunflower (*Helianthus annuus* L.). *J. Plant Biochem. Biot.* **7**: 107-110.

Kalyaeva, M. A., Zacharchenko, N. S., Doronina, N. V., Rukavtsova, E. B., Ivanova, E. G., Alekseeva, V. V., Trotsenko, Y. A. and Bur'yanov, Y. I. (2001) Plant growth and morphogenesis *in vitro* is promoted by associative methylotrophic bacteria. *Russ. Plant Physiol.* **48**:514-517.

Kalyaeva, M. A., Ivanova, E. G., Doronina, N. V., Zakharchenko, N. S., Trotsenko, Y. A. and Buryanov, Y. I. (2003) The effect of aerobic methylotrophic bacteria on the *in vitro* morphogenesis of soft wheat (*Triticum aestivum*). *Russ. Plant Physiol.* **50**:513-517.

Kandpal, R. P. and Rao, N. A. (1985). Changes in levels of polyamines in ragi seedlings during water stress. *Biochem. Int.* **11**: 365-370.

Kantety, R. V., Zeng, X. P., Bennetzen, J. L. and Zehr, B. E. (1995). Assessment of genetic diversity in dent and popcorn *(Zea mays* L.) inbred lines using inter-simple sequence repeat (ISSR) amplification. *Mol. Breed.* **1**: 365-373.

Karban, R., Agrawal, A. A. and Mangel, M. (1997). The benefits of induced defence against herbivores. *Ecology* **78**: 1351-1355.

Karp, A. (1991). On the current understanding of clonal variation. B. J. Miflin. *Oxford Surveys of Plant Molecular and Cell Biology.* **7.:** 1-58.

Karp, A., Seberg, O. and Buiatti, M. (1996). Molecular techniques in the assessment of botanical diversity. *Ann. Bot.* **78**: 143-149.

Kartha, K. K. (1981). Meristem culture and cryopreservation methods and applications. T. A. Thorpe. *Plant Tissue Culture: Methods and Applications in Agriculture.* New York, Academic Press: 181 - 211.

Kasahara, J., Nakamura, T. and Yoneyama, Y. (1973). Graft-induced variation in red peppers. *Rec. Adv. Breed.* **13**: 73-89.

Kaufman, P., Wu, L.-L., Brock, T. and Kim, D. (1995). Hormones and the orientation of growth. P. Davies. *Plant Hormones: Biology, Biochemistry and Molecular Biology.* Dordrecht. Kluwer Academic Publishers: 547-571.

Kaul, K. (1986). Establishment of long-term callus cultures from mature white pine (*Pinus strobus,* Pinaceae). *Am. J. Bot.***73**: 242 - 245.

Kaur, K., Verma , B. and Kant, U. (1998). Plants obtained from the Khair tree (*Acacia catechu* Willd.) using mature nodal segments. *Plant Cell Rep.* **17**(5): 427 - 429.

Kawata, M., Ohmiya, A., Shimamoto, Y., Oono, K., Takaiwa, F. (1995). Structural changes in plastid DNA of rice (*Oryza sativa* L.) during tissue culture. *Theor. Appl. Genet.* **90**: 364-371.

Kayser, M. e. a. (2000). Characteristics and frequency of germline mutations at microsatellite loci from the human Y chromosome, as revealed by direct observation in father/son pairs. *Am. J. Hum. Genet.* **66**: 1580-1588.

Kevers, C., Hausman, J. F., Faivre-Rampant, O., Evers, D. and Gaspar, T. (1997.). Hormonal control of adventitious rooting: progress and questions. *Angew. Bot.* **71**: 71-79.

Khuri, S. and Moorby, J. (1995). Investigations into the role of sucrose in potato cv. *Estima microtuber* production *in vitro*. *Ann. Bot.* **75**: 295-303.

Kim, M.-S., Schumann, C. M. and Klopfenstein, N. B. (1997). Effects of thidiazuron and benzyladenine on axillary shoot proliferation of three ash (*Fraxinus pennsylvanica* Marsh.) clones. *Plant Cell. Tiss. Org. Cult.* **48**: 45-52.

Kimura, M. (1983). The Neutral Theory of Evolution. Cambridge University Press.

Kirk, J. T. O. and Tilney-Bassett, R. A. E. (1978). The plastids: Their Chemistry, Structure, Growth and Inheritance. New York, Elsevier/ North-Holland Biomedical Press.

Klein, B. and Bopp, M. (1971). Effect of activated charcoal in agar on the culture of lower plants. *Nature* **230**: 474.

Kleinschmit, J. (1999). Breeding strategies for hardwoods: oak, cherry and birch. G. C. Douglas. *Strategies for Improvement of Forest Trees*. Dublin, COFORD.

Klekowski, E. J. (1988). Mutation, Developmental Selection, and Plant Evolution. New York., Columbia University Press.

Klekowski, E. J. and Godfrey, P. J. (1989). Aging and mutation in plants. *Nature* **340**: 389-391.

Knox, J. P. and Wareing, P. F. (1984). Apical dominance in *Phaseolus vulgaris* L.: The possible roles of absicic and indole-3-acetic acid. *J. exp. Bot.* **35**: 239-244.

Kojima, T., Nagaoka, T., Noda, K. and Ogihara, Y. (1998). Genetic linkage map of ISSR and RAPD markers in Einkorn wheat in relation to that of RFLP markers. *Theor. Appl. Genet.* **96**: 37-45.

Kolzowski, T. T. and Pallardy, S. G. (1997a). Physiology of Woody Plants. San Diego, Academic Press.

Kolzowski, T. T. and Pallardy, S. G. (1997b). Growth control in Woody Plants. San Diego, Academic Press.

Konigshofer, H. (1990). Polyamine content in needles, shoot axes, buds and xylem exudates of mature spruce trees (*Picea abies* L. Karst.). *J. Plant Physiol.***136**: 377-380.

Konwar, B. and Coutts, R. (1990). Rapid regeneration of sugar beet (*Beta vulgaris* L.) plants from *in vitro* cultures. H. Nijkamp, L. Van Der Plas and V. Aartrijk. *Progress in Plant Cellular and Molecular Biology. Proceedings of the VIIth International Congress on Plant Tissue & Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990.* Dortrecht, Netherlands., Kluwer Academic Publishers: 114-118.

Kosower, N. S. and Kosower, E. M. (1978). The glutathione status of cells. *Int. Rev. Cytol.* **54**: 109-160.

Kovalchuk, I., Kovalchuk, O. and Hohn, B. (2000b). Genome-wide variation of the somatic mutation frequency in transgenic plants. . *EMBO J.* **19**: 4431-4438.

Kovalchuk, O., Dubrova, Y. E., Arkhipov, A., Hohn, B. and Kovalchuk, I. (2000a). Wheat mutation after Chernobyl. *Nature* **407**: 583.

Kozai, T. and Iwanami, Y. (1988). Effects of CO_2 enrichment and sucrose concentration under high photon fluxes on plantlet growth of carnation (*Dianthus caryophyllus* L.) in tissue culture during the preparation stage. *J. Jpn. Soc. Hortic. Sci.* **57**: 279-288.

Kozai, T., Koyama, Y. and Watanabe, I. (1988). Multiplication of potato plantlets *in vitro* with sugar free medium under high photosynthetic photon flux. *Acta Hort.* **230**: 121-127.

Kozai, T., Oki, H. and Fujiwara, K. (1987). Effects of CO_2 enrichment and sucrose concentration under high photosynthetic photon fluxes on growth of tissue-cultured *Cymbidium* plantlets during the

preparation stage. Proceedings of Symposium Florizel: Plant Microporpagation in the Horticultural Industry, Arlon, Belgium.

Kracke, H. and Cristoferi, G. (1983). Effect of IBA and NAA treatments on the endogenous hormones in grapevine rootstocks hardwood cuttings. *Acta Hort.* **137**: 95-101.

Kramer, P. J. and Kozlowski, T. T. (1979). Physiology of woody plants. London, Academic press Inc.

Krasnosselskaya, J. A. and Richter, A. A. (1942). Transport of break of winter dormancy of buds along branches of woody plants. *Compt. Rend. (Doklady) Acad. Sci. USSR* **35**(6): 184-186.

Kretzschmar, U. and Ewald, D. (1994). Vegetative propagation of 140 year-old *Larix decidua* trees by different *in vitro* techniques. *Plant Physiol.***144**: 627 - 630.

Krikorian, A. (1988). Plant tissue culture: Perceptions and realities. *Proc. Indian Acad. Science (Plant Science).* **98**: 425-464.

Krikorian, A. (1995). Hormones in tissue culture and micropropagation. P. Davies. *Plant Hormones: Biology, Biochemistry and Molecular Biology.* Dordrecht, Kluwer Academic Publishers: 774-796.

Krikorian, A. D. and Berquam, D. L. (1969). Plant cell and tissue cultures: The role of Haberlandt. *Bot. Rev.* **35**: 59 - 88.

Kulpa, J. M., Galsky, A. G., Lipetz, P. and Stephens, R. (1985). Polyamines and crown gall tumour growth. *Plant Cell Rep.* **7**: 138-141.

Kumar, A., Altabella, T., Taylor, M. A. and Tiburcio, A. F. (1997). Recent advances in polyamine research. *Trends Plant Sci.* **2**: 124-130.

Kunneman, B. (1995). Hardwood and softwood cuttings: Factors affecting rootability. *Comb. Proc. Int. Plant. Prop. Soc.* **45**: 157-160.

Kunneman, B. and Otten, G. (1994). Bodemwarmte stimuleert beworteling winterstekken van laanbomen. *De Boomkwekerij* **47**: 18-21.

Kunnemann-Kooji (1984). Inventarisatie van de toepassingsmogelijkheden van weefselkweek vorr de vegatatieve vermeerdering van houtige boomkwekerijgewassen. The Netherlands, Department of Horticulture, Agricultural University Wageningen.

Kushad, M. M. and Dumbroff, E. B. (1991). Metabolic and physiological relationships between the polyamine and ethylene biosynthetic pathways. R. D. Slocum and H. E. Flores. *The Biochemistry and Physiology of Polyamines in Plants.* Boca Raton, Florida, CRC Press: pp 78-89.

Lakshmi, S. and Rhani, S. (1985). *In vitro* propagation of *Eucalyptus grandis* L. by tissue culture. *Plant Cell Rep.* **4**: 63-65.

Lally, M. and Fennessey, J. (2002). Ireland. J. Turok, G. Eriksson, K. Russell and S. Borelli. *Noble Hardwoods Network, Report of the fourth meeting, 4-6 September 1999, Gmunden, Austria, and the fifth meeting, 17-19 May 2001, Blessington, Ireland.* Rome, Italy, International Plant Genetic Resources Institute.

Lang, G. A. (1987). Dormancy: A new universal terminology. *HortScience* **22**: 817-820.

Lang, G. A., Early, J. D., Arroyave, N. G., Darnell, R. L., Martin, G. C. and Stutte, G. W. (1985). Dormancy: Toward a universal terminology. *HortScience* **20**: 809-811.

Lang, G. A., Early, J. D., Martin, G. C. and Darnell, R. L. (1987). Endo-, para-, and ecodormancy: Physiological terminology and classification for dormancy research. *Hort Science* **22**: 371-377.

Langens-Gerrits, M., Albers, M. and De Klerk, G.-J. (1998). Hot water treatment before tissue culture reduces initial contamination in *Lilium* and *Acer*. *Plant Cell. Tiss. Org. Cult.* **52**: 75-77.

Larkin, P. J. and Scowcroft, S. C. (1981). Somaclonal variation - a novel source of variability from cell cultures for crop improvement. *Theor. Appl. Gen.* **60**: 197-214.

Law, R. D. and Suttle, J. C. (2003). Transient decreases in methylation at 5 -CCGG-3 sequences in potato (*Solanum tuberosum* L.) meristem DNA during progression of tubers through dormancy precede the resumption of sprout growth. *Plant Mol. Biol.* **51**: 437–447.

Leforestier, F., Courtois-Gras, M. and Joseph, C. (1990). Micropropagation of *Fraxinus excelsior*. Proceedings of seventh International Congress on Plant Tissue and Cell Culture, Amsterdam.

Leforestier, F., Courtois-Gras, M. and Joseph, C. (1991). Etude comparative de la micropropagation de quelques especes de *Fraxinus*. *Acta Hort.* **289**: 215.

Lefort, F., Brachet, S., Frascaria-Lacoste, N., Edwards, K. J. and Douglas, G. C. (1999). Identification and characterisation of microsatellite loci in ash (*Fraxinus excelsior* L.) and their conservation in the olive family (Oleaceae). *Mol. Ecol.* **8**: 1088-1089.

Leifert, C., Murphy, K. P. and Lumsden, P. J. (1995). Mineral and carbohydrate nutrition of plant cell and tissue cultures. *Crit. Rev. Plant Sci.* **14**: 83-109.

Leroy, X. J., Leon, K. and Branchard, M. (2000a). Plant genomic instability detected by microsatellite - primers. *Electr. J. Biotech. [online].* **3**: [cited 6 April 2004].

Leroy, X. J. and Leon, K. (2000b). A rapid method for detection of plant genomic instability using anchored microsatellite primers. *Plant Mol. Biol. Rep.* **18**: 283a-283g.

Leva, A. R. and Muleo, R. (1993). Effects of "media osmotic agents" on the growth and morphogensis of *Actinidia deliciosa* c.v. "Hayward" callus. *In Vitro Cell. Devel. Biol.-Plant.* **29**: 59-64.

Leva, A. R., Petruccelli, G. and Bartolini, G. (1994). Mannitol *in vitro* culture of *Olea europaea* L. (cv. Maurino). *Acta Hort.* **356**: 43-46.

Levinson, G. and Gutman, G. A. (1987). Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Mol. Biol. Evol.* **4**: 203-221.

Lewis, W. H., Oliver, R. L. and Luikart, T. K. (1971). Multiple genotypes in individuals of *Claytonia virginica*. *Science* **172**: 564-565.

Leyser, O. (2003). Regulation of shoot branching by auxin. *Trends Plant Sci.* **8**: 541-545

Li, C. J., Guevara, E., Gerrera, J. and Bangerth, F. (1995). Effect of apex excision and replacement by 1-napthylacetic acid on cytokinin concentration and apical dominance in pea plants. *Physiol Plant.* **94**: 465-469.

Li, C. Y., Viherä-Aarnio, A., Puhakainen, T., Junttila, O., Heino, P. and Palva, E. T. (2003). Ecotype-dependent control of growth, dormancy and freezing tolerance under seasonal changes in *Betula pendula* Roth. *Trees- Struct. Funct.* **17**: 127–132.

Li, W.-H. (1997). Molecular Evolution. Massachusetts, U. S. A., Sinauer Associates, Inc., Publishers.

Libby, W. J. (1973). Domestication strategies for forest trees. *Can. J. Forest Res.* **3**: 265-276.

Linacero, R. and Vazquez, A. M. (1992). Genetic analysis of chlorophyll-deficient somaclonal variants in rye. *Genome* **35**: 981-984.

Linsmaier, E. M. and Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant.* **18**: 100-127.

Lipavska, H. and Vreugdenhil, D. (1996). Uptake of mannitol from the media by the *in vitro* growth plants. *Plant Cell. Tiss. Org. Cult.* **45**: 103-107.

Liscum, E. (1999). Amplified Fragment Length Polymorphism: Studies on Plant Development. M. A. Innis, D. H. Gelfand and J. J. Sninsky. *PCR Applications*, Academic Press: 505-520.

Litz, R. E. and Jaiswal, V. S. (1991). Micropropagation of tropical and subtropical fruits. P. C. Debergh and R. H. Zimmerman. *Micropropagation: Technology and Application.* Netherlands, Kluwer Academic Publishers.

Liu, D., Faust, M., Millard, M. M., Line, M. J. and Stutte, G. W. (1993). States of water in summer-dormant apple buds determined by proton magnetic resonance imaging. *J. Am. Soc. Hort. Sci.* **118**: 632-637.

Liu, J. and Reid, D. (1992). Adventitious rooting in hypocotyls of sunflower (*Helianthus annus*) seedlings. IV. The role of changes in endogenous free and conjugated indole-3-acetic acid. *Physiol. Plant.* **86**: 285-292.

Liu, K., Fu, H., Bei, Q. and Luan, S. (2000). Inward potassium channel in guard cells as a target for polyamine regulation of stomatal movements. *Plant Physiol.* **124**: 1315-1326.

Lloyd, G. and McCown, B. (1980). Use of microculture for production and improvement of *Rhododendron* spp. *HortScience* **15**: 416.

Lloyd, G. and McCown, B. (1981). Commercially feasible micropropagation of mountain Laurel, *Kalmia latifolia*, by use of shoot tip culture. *International Plant Propagation Society* **30**: 421 - 427.

Loxdale, H. D. and Lushai, G. (2003). Rapid changes in clonal lines: the death of a 'sacred cow'. *Biol. J. Linn. Soc.* **79**: 3-16.

Lucchesini, M. and Mensuali-Sodi, A. (2004). Influence of medium composition and vessel ventilation on in vitro propagation of *Phillyrea latifolia* L. *Scientia Hort.* **100**: 117-125.

Lyrene, P. (1981). Juvenility and production of fast-rooting cuttings from blueberry shoot cultures. *J. Amer. Soc. Hort. Sci.* **106**: 396-398.

Lystaght, L. (2000). The right trees please! *Heritage Outlook* (1).

MacDonald, B. (1989). Practical woody plant propagation for nursery growers. Portland, Oregan., Timber Press,.

Magalewski, R. L. and Hackett, W. P. (1979). Cutting propagation of *Eucalyptus ficifolia* using cytokinin-induced basal trunk sprouts. *Proc. Int. Plant. Prop. Soc.* **29**: 118.

Maldiney, R., Pelese, F., Pilate, B., Sotta, B., Sossountzov, L. and Miginiac, E. (1986). Endogenous levels of abscisic acid, indole-3-acetic acid, zeatin and zeatin-riboside during the course of adventitious root formation in cuttings of *Craigella* and *Graigella* lateral suppressor tomatoes. *Physiol. Plant.* **68**: 426-430.

Mante, S., Scorza, R. and Cordts, J. M. (1989). Plant regeneration from cotyledons of *Prunus domestica*, and *Prunus cerasus*. *Plant Cell. Tiss. Org. Cult.***19**: 1-11.

Manzanera, J. A., Bueno, M. A. and Pardos, J. A. (1996). *Quercus robur* L. (Pedunculate Oak). Bajaj. *Biotechnology in Agriculture and Forestry. Trees 4.* Berlin, Springer-Verlag. **35:** 321-341.

Marcotrigiano, M. (1985). Synthesising plant chimeras as a source of new phenotypes. *Int. Plant. Prop. Soc.* **35**: 582-586.

Marcotrigiano, M. (1991). Understanding foliar variegation as it relates to propagation. *Comb. Proc. Int. Plant. Prop. Soc.* **41**: 410-415.

Marin, J. A. and Gella, R. (1987). Aclimatization of the micropropagated cherry rootstock "Masto de Montanana" (*Prunus cerasus* L.). *Acta Hort.* **212**: 603 - 606.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., Brattain, M. and Willson, J. K. V. (1995). Inactivation of the type II TGF-b receptor in colon cancer cells with microsatellite instability. *Science* **268**: 1336-1338.

Marks, T. (1996). The role of the shoot apex in controlling rhizogenesis *in vitro*. *Plant Growth Regul.* **20**: 57-60.

Marks, T. R. and Myers, P. E. (1992). Effect of explant location upon early culture development *in vitro*. *J. Hort. Sci.* **67**: 583-591.

Marks, T. R. and Myers, P. E. (1994). Physiological variability arising from *in vitro* culture is induced by shoot selection and manipulation strategies. *J. Hort. Sci.* **69**: 1-9.

Marks, T. R., Simpson, S. E., Beckham, C. F. and Dobson, H. E. (1987). Micropropagation of hardy ornamentals. *East Malling Research Station Report . 1986.*: 31-34.

Martin-Tanguy, J. (1985). The occurrence and possible function of hydroxycinnamoyl acid amides in plants. *Plant Growth Regul.* **3**: 381 - 399.

Martin-Tanguy, J., Martin, C., Paynot, M. and Rossin, N. (1988). Effect of hormone treatment on growth bud formation and free amine and hydroxycinnamoyl putrescine levels in leaf explant of *Nicotiana tabacum* cultivated *in vitro*. *Plant Physiol.* **88**: 600-604.

McAfee, B., White, E., Pelcher, L. and Lapp, M. (1993). Root induction in pine (*Pinus*) and larch (*Larix*) spp. using *Agrobacterium rhizogenes*. *Plant Cell. Tiss. Org. Cult.***34**: 53-62.

McClelland, M., Smith, M. and Carothers, Z. (1990). The effects of *in vitro* and *ex vitro* root initiation on subsequent microcutting root quality in three woody plants. *Plant Cell. Tiss. Org. Cult.* **23**: 115-123.

McComb, J. and Bennett, I. (1986). Eucalyptus (*Eucalyptus* spp.). Y. Bajaj. *Biotechnology in agriculture and forestry. Vol 1. Trees 1.* Berlin, Springer Verlag: 340-362.

McComb, J. A. and Bennett, I. J. (1982). Vegetative propagation of *Eucalyptus* using tissue culture and its application to forest improvement in Western Australia. Proceedings of the 5th International Congress on Plant Tissue Cell Culture., Tokyo, Japan, Japanese Association of Plant Tissue Culture.

McCown, B. a. L., G. (1981). Woody plant medium (WPM) - a mineral nutrient formulation for microculture of woody plant species. *HortScience* **16**: 453.

McCown, B. H. and Sellmer, J. C. (1987). General media and vessels suitable for woodplant culture. J. M. Bonja and D. J. Durzan. *Cell and Tissue Culture in Forestry.* Dordrecht, Martinus Nijhoff. **1:** 4-16.

McCown, B. H. and Sellmer, J. C. (1987). General media and vessels suitable for woodyplant culture. J. M. Bonga and D. J. Durzan. *Cell and Tissue Culture in Forestry.* Dordrecht, Martinus Nijhoff Publishers. **1:** 4-16.

McCown, D. D. and McCown, B. H. (1987). North American Hardwoods. J. M. Bonga and D. J. Durzan. *Cell and Tissue Culture in Forestry.* Dordrect, Martinus Nijhoff. **3:** 247 - 260.

McEvoy, T. (1979). Forestry. D. A. Gillmor. *Irish Resources and Land Use.* Dublin, Institute of Public Administration.

Mederos-Molina, S. and Trujillo, M. I. (1999). Elimination of browning exudate and *in vitro* development of shoots in *Pistacia vera* L. cv. mateur and *Pistacia atlantica* Desf. culture. *Acta Soc. Bot. Poloniae* **68**: 21-24.

Meier, K. and Reuther, G. (1994) Factors controlling micropropagation of mature *Fagus sylvatica*. *Plant Cell. Tiss. Org. Cult.* **39**:231-238.

Meier-Dinkel, A. (1987). *In vitro* vermehrung und weiterkultur von stieleiche (*Quercus robur* L.) und Traubeneiche (*Quercus petraea* (Matt.) Liebl.). *Allg. Forst. Jagdztg* **158**: 199-204.

Meier-Dinkel, A. (1991). Recovery of juvenile characteristics through *in vitro* propagation of mature fast growing birch hybrids. M. R. Ahuja. *Woody Plant Biotechnology.* New York, Plenum Press: 345-346.

Meier-Dinkel, A., Becker, B. and Duckstein, D. (1993). Micropropagation and *ex vitro* rooting of several clones of late flushing *Quercus robur* L. *Ann. Sci. For.* **50**: 319-322.

Meier-Dinkel, A. and Kleinschmit, J. (1990). Aging in tree species: Present knowledge. R. Rodríguez, T. R. Sánchez and D. J. Durzan. *Plant Aging: Basic and Applied Approaches*. New York, Plenum Press: 51-63.

Mencuccini, M. (2002). Effect of medium darkening on *in vitro* rooting capability and rooting seasonality of olive (*Olea europaea* L.) cultivars. *Scientia Hort.* **1845**: 1-11.

Menges, M., Hennig, L., Gruissem, W. and Murray, J. A. (2002). Cell cycle-regulated gene expression in *Arabidopsis*. *J. Biol. Chem.* **277**: 41987–42002.

Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B. and Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the human suppresser p16/CDKN2/MTS1 in human cancers. *Nature Medicine* **1**: 686-692.

Micheli, F., Sundberg, B., Goldberg, R. and Richard, L. (2000). Radial distribution pattern of pectin methylesterases across the cambial region of hybrid aspen at activity and dormancy. *Plant Physiol.* **124**: 191–200.

Micheli, M., Mencuccini, M. and Standardi, A. (1998). Encapsulation of *in vitro* proliferated buds of Olive. *Adv. Hortic. Sci.* **12**: 163 - 168.

Michod, R. E. (1999.). Darwinian Dynamics: Evolutionary Transitions in Fitness and Individuality. Princeton, NJ., Princeton University Press.

Miller, C. R., Joyce, P. and Waits, L. P. (2002). Assessing allelic dropout and genotyping reliability using maximum likelihood. *Genetics* **160**: 357-366.

Mission, J. P. and Giot-Wirgot (1984). Rajeunissement d'un clone de *Thuja* envue de sa multiplication *in vitro*. *Ann. Rech. Sylvicoles*. Fontainebleau, AFOCEL: 187 - 197.

Mitchell, F. and Ryan, M. (1998). Reading the Irish Landscape. Dublin, Townhouse and Country House.

Mitsuhashi-Kato, M., Mishibaoka, H. and Shimokoriyama, M. (1978). Anatomical and physiological aspects of developmental processes of adventitious root formation. *Plant Cell Physiol.* **19**: 393-400.

Mohammed, A. and Eriksen, E. (1974). Root formation in pea cuttings. IV. Further studies on the influence of indole-3-acetic acid at different developmental stages. *Physiol. Plant.***32**: 94-96.

Mok, M. C., Mok, D. W. S., Armstrong, D. J., Shudo, K., Isogai, Y. and Okamoto, T. (1982). Cytokinin activity of N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron). *Phytochemistry* **21**: 1509-1511.

Mokotedi, O., Watt, P., Pammenter, N. and Blakeway, F. (2000). *In Vitro* Rooting and Subsequent Survival of Two Clones of a Cold-tolerant *Eucalyptus grandis* Hill ex Maid. X *E. nitens* (Deane & Maid.) Hybrid. *HortScience* **35**(6): 1163-1165.

Moncousin, C., Favre, J. and Gaspar, T. (1988). Changes in peroxidase activity and endogenous IAA levels during adventitious root formation in vine cuttings. M. Kutacek, R. Bandurski and J. Krekule. *Biochemistry of Auxin in Plants*. The Hague, SPB Academic Publishing: 331-338.

Morand, M. E., Brachet, S., Rossignol, P., Dufour, J. and Frascaria-Lacoste, N. (2002). A generalized heterozygote deficiency assessed with microsatellites in French common ash populations. *Mol. Ecol.* **11**: 377 - 385.

Morand, M. E., Vedel, F., Raquin, C. and Frascaria-Lacoste, N. (2001). A rapid and efficient method for isolation of mitochondrial DNA from angiosperm tree species. Application to the restriction fragment length polymorphism distinction between European and American ashes. *Mol. Ecol. Notes* **1**: 67-70.

Morand-Prieur, M. E., Raquin, C., Shykoff, J. A. and Frascaria-Lacoste, N. (2003). Males outcompete hermaphrodites for seed siring sucess in controlled crosses in the polygamous *Fraxinus excelsior* (Oleaceae). *Am. J. Bot.* **90**: 949-953.

Morand-Prieur, M. E., Vedel, F., Raquin, C., Brachet, S., Sihachakr, D. and Frascaria-Lacoste, N. (2002). Maternal inheritance of a chloroplast microsatellite marker in controlled hybrids between *Fraxinus excelsior* and *Fraxinus angustifolia*. *Mol. Ecol.* **11**: 613-617.

Morard, P. and Henry, M. (1998). Optimization of the mineral composition of *in vitro* culture media. *J. Plant Nutr.* **21**: 1565-1576.

Morel, G. and Martin, C. (1952). Guerison de dahlias atteints d'une maladie a virus. *C. R. Hebd. Seane. Acad. Sci. Paris.* **235**: 1342 - 1325.

Morel, G. M. (1960). Producing virus free *Cymbidium*. *Am. Orchid Soc. Bull.* **29**: 495 - 497.

Morelli, G. and Ruberti, I. (2000). Shade avoidance responses: driving auxin along lateral routes. *Plant Physiol* **122**: 621-626.

Morgan, T. H. (1901). Regeneration. London, Macmillan.

Morikawa, H. and Yamada, Y. (1992). Protoplast fusion. M. W. Fowler, G. S. Warren and M. Moo-Young. *Plant biotechnology.* Oxford, Pergamon Press.: 199-222.

Mott, R. L. (1986). Trees. B. V. Conger. *Cloning Agricultural Plants Via In Vitro Techniques.* Florida, CRC Press.

Mullins, M. G., Nair, Y. and Sampet, P. (1979). Rejuvenation *in vitro*: Induction of juvenile characters in adult clone of *Vitis vinifera* L. *Ann. Bot.* **44**: 623 - 627.

Munthali, M. T., Newbury, H. J. and Ford-Llyod, B. V. (1996). The detection of somaclonal variants of beet using RAPD. *Plant Cell Rep.* **15**: 474-478.

Murai, Y., Harada, H. and Yumaji, Y. (1995). Cultivar differences in shoot proliferation and rooting of Apricot (*Prunus armeniaca* L.) *in vitro*. *Comb. Proc. Int. Plant. Prop. Soc.* **45**: 631-634.

Murashige, T. (1974). Plant propagation through tissue culture. *HortScience* **9**: 2 - 3.

Murashige, T. (1974). Plant propagation throuh tissue culture. *Ann. Rev. Plant Physiol.* **25**: 135 - 166.

Murashige, T. and Huang, L. C. (1987). Cloning Plants by Tissue Culture: The Early Years, Current Status and Future Prospects. *Acta Hort.* **212**: 35 - 42.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tabocco tissue cultures. *Physiol. Plant.* **15**: 473 - 497.

Murray, J., Sánchez, M., Smith, A., Hackett, W. (1994). Differential competence for adventitious root formation in histologically similar cell types. T. Davies and B. Haissig. *Biology of adventitious root formation.* New York, Plenum Press: 99-109.

Murray, J. R., Smith, A. G. and Hackett, W. P. (1994). Differential dihydroflavonol reductase transcription and anthocyanin pigmentation in the juvenile and mature phase of Ivy (*Hedera helix* L.). *Planta* **194**: 102 - 109.

Nadel, B., Altman, A., Pleban, S., Kocks, R. and Huttermann, A. (1991). *In vitro* developement of mature *Fagus sylvatica* L. buds. II. Seasonal changes in the response to plant growth regulators. *J. Plant Physiol.* **138**: 136-141.

Nagaoka, T. and Ogihara, Y. (1997). Applicability of inter-simple sequence repeat poly-morphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. *Theor. Appl. Genet.* **94**: 597-602.

Nahlawi, N. and Howard, B. (1973). The effects of duration of the propagation period and frequency of auxin treatment on the response of plum hardwood cuttings to IBA. *J. Hort. Sci.* **48**: 169-174.

Nas, M. N. and Read, P. E. (2000). Micropropagation of hybrid hazelnut: medium composition, physical state and iron source affect shoot morphogenesis, multiplication and explant vitality. *Proceedings of the Fifth International Congress on Hazelnut. Acta Hort.* **556**: 251-258.

Nas, M. N. and Read, P. E. (2004). A hypothesis for the development of a defined tissue culture medium of higher plants and micropropagation of hazelnuts. *Scientia Hort.* **101**: 189-200.

Navarrete, N. E., Van Sambeek, J. W., Preece, J. E. and Gaffney, G. R. (1989). Improved micropropagation of white ash *Fraxinus americana* L. Proceedings of the 7th Central Hardwood Conference, Southern Illinois University at Carbondale, General Technical Report No. NC-132 of the North Central Forest Experiment Station, USDA Forest Service.

Navidi, W., Arnheim, N. and Waterman, M. S. (1992). A multipletubes approach for accurate genotyping of very small DNA samples by using PCR: statistical considerations. *Am. J. Hum. Genet.* **50**: 347-359.

Nawa, S. and Yamada, M. A. (1967). Genetic effects of DNA in *Ephestia*. *Natl. Inst. Genet. Japan Ann. Rep.* **17**: 48-49.

Nawa, S. and Yamada, M. A. (1968). Hereditary changes in *Ephestia* after treatment with DNA. *Genetics* **58**: 573-584.

Nee, C. C. (1987). Overcoming bud dormancy with hydrogen cynamide: timing and mechanism. Corvallis, OR, Oregon State University: 144.

Nei, M. (1973). Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. USA.* **70**: 3321-3323.

Nei, M. (1975). Molecular Population Genetics and Evolution. Amsterdam, North Holland.

Nei, M. (1987). Molecular Evolutionary Genetics. New York, Columbia University Press.

Nei, M. and Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* **76**: 5269-5273.

Németh, G. (1979). Benzyladenine-stimulated rooting in fruit-tree rootstocks cultured *in vitro*. *Z. Pflanzenphysiol.* **95**: 389-396.

Németh, G. (1986). Induction of Rooting. Y. P. S. Bajaj. *Biotechnology in Agriculture and Forestry. Trees 1.* Berlin Heidelberg, Springer-Verlag.

Ng, N. Q. and Ng, S. Y. C. (2002). Genetic Resources and Conservation. R. J. Hillocks, J. M. Thresh and A. C. Bellotti. *Cassava: Biology, Production and Utilization*, CAB International.

Nickerson, P. (1995). Rooting lilacs from softwood cuttings. *Comb. Proc. Int. Plant. Prop. Soc.* **45**: 502-503.

Niemirowicz-Szczytt, K., Faris, N. M., Rucinska, M. and Nikolova, V. (2000) Conservation and storage of a haploid cucumber (*Cucumis sativus* L.) collection under *in vitro* conditions. *Plant Cell Rep.* **19**:311-314.

Nitsch, J. P. and Strain, G. C. (1969). Effet de diverses cytokinines sur le brunissement d'explants de canne a sucre. *Compt. Rend. Acad. Sci. Paris* **268**: 806 - 809.

Noguchi, T., Hirata, Y. and Yagishita, N. (1992). Intervarietal and interspecific chimera formation by *in vitro* graft-culture methods in *Brassica. Theor. Appl. Gen.* **83**: 127-132.

Nomura, K., Matsumoto, S., Masuda, K. and Inoue, M. (1998). Reduced glutathione promotes callus growth and shoot developement in a shoot tip culture of apple root stock M26. *Plant Cell Rep* **17**: 597-600.

Nordstrom, A. and Eliasson, L. (1984). Regulation of root formation by auxin-ethylene interaction in pea stem cuttings. *Physiol. Plant.***61**: 298-302.

Normanly, J., Slovin, J. P. and Cohen, J. D. (1995.). Rethinking auxin biosynthesis and metabolism. *Plant Physiol.* **107**: 323-329.

Norton, M. E. and Norton, C. R. (1986). Explant origin as a determinant of *in vitro* shoot proliferation in *Prunus* and *Spirea. J. Hort. Sci.* **61**: 43-48.

Oh, Y. and Mao, L. (1999). Microsatellite Protocols. M. A. Innis, D. H. Gelfand and J. J. Sninsky. *PCR Applications*, Academic Press: 355-364.

Ohta, T. a. K., M. (1973). A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. *Genet. Res.* **22**: 201-204.

Ohta, Y. (1970). A variant found in the progeny from grafting in *Capsicum annuum. Natl. Inst. Genet. Japan Ann. Rep.* **20**: 34-35.

Ohta, Y. (1977a). Heredity changes induced by grafting and by DNA application and their implication in Capsicum breeding. E. Pochard. *"Capsicum 77" Proc. III Eucarpia Meeting on Genetics and Breeding of Capsicum*. France. Avignon-Montfavet: 147-153.

Ohta, Y. (1977b). Heredity changes induced by grafting. *Sci Amer.* **7**: 100-113.

Ohta, Y. (1977c). Graftoduction in *Capsicum annuum* L. and an interpretation on the mechanism. *Japan J. Breed.* **27**: 130-131.

Ohta, Y. (1991). Graft-transformation, the mechanism for graft induced genetic changes in higher plants. *Euphytica* **55**: 91-99.

Ohta, Y. and Choung, P. V. (1975a). Hereditary changes in *Capsicum annuum* L. I. Induced by ordinary grafting. *Euphytica* **24**: 355-368.

Ohta, Y. and Choung, P. V. (1975b). Hereditary changes in *Capsicum annuum* L. II. Induced by virus-inoculated grafting. *Euphytica* **24**: 605-611.

Okuba, H. (2000). Growth cycle and dormancy in plants. J. D. Viemont and J. Crabbe. *Dormancy in Plants – From Whole Plant Behavior to Cellular Control*, CABI: 1–22.

O'Neill, J. J. M. and Mitchell, D. T. (1999). *Glomus claroideum*, an arbuscular mycorrhizal fungus new to Ireland, and its distribution in an Irish tree nursery. *Biology and Environment: Proceedings of the Royal Irish Academy.* **99B**(3): 197 - 203.

Or, E., Eyal, Y. and Ogrodovitch, A. (2000). The transduction of the signal for grape bud dormancy breaking induced by hydrogen cyanamide may involve the SNF-like protein kinase GDBRPK. *Plant Mol. Biol.* **43**: 483–494.

Otto, S. P. and Hastings, I. M. (1998.). Mutation and selection within the individual. *Genetics* **102/103**: 507-524.

Owen, H. R., Wengerd, D. and Miller, A. R. (1991). Culture medium pH influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage methods. *Plant Cell Rep.* **10**: 583-586.

Page, R. D. M. and Holmes, E. C. (1998). Molecular Evolution. Oxford, U. K., Blackwell Science Ltd.

Palumbi, S. R. (1996). Nucleic Acids II: The Polymerase Chain Reaction. D. M. Hillis, C. Moritz and B. K. Mable. *Molecular Systematics*. USA, Sinauer Associates: 205-248.

Pandey, K. K. (1976). Genetic transformation and 'graft hybridization' in flowering plants. *Theor. Appl. Genet.* **47**: 299-302.

Pandey, K. K. (1985). Gene movement and genetic manipulation. M. Freeling. *Plant Genetics.* New York., Alan R. Liss, Inc.: 235-249.

Parkinson, M., Prendergast, M. and Sayegh, A. J. (1996). Sterilisation of explants and cultures with sodium dichloroisocyanurate. *Plant Growth Regul.* **20**: 61-66.

Parmentier, C. M., Rowland, L. J. and Line, M. J. (1998). Water status in relation to maintenance and release from dormancy in blueberry flower buds. *J. Amer. Soc. Hort. Sci.* **123**(5): 762 - 769.

Pawlicki, N. and Welander, M. I. (1995). nfluence of carbohydrate source, auxin concentration and time of exposure on adventitious rooting of the apple root stock Jork 9. *Plant Sci.* **106**: 167-176.

Peltier, J. P., Marigo, D. and Marigo, G. (1997). Involvement of malate and mannitol in the diurnal regulation of the water status in members of the Oleaceae. *Trees* **12**: 27-34.

Penalver, R., Duranvila, N. and Lopez, M. M. (1994) Characterisation and pathogenicity of bacteria from shoot tips of globe artichoke (*Cynara scolymus* L.). *Ann. Appl. Biol.* **125**:501-513.

Penner, G. (1996). RAPD Analysis of Plant Genomes. P. P. Jauhar. *Methods of Genome Analysis in Plants.* New York, CRC Press: 251-268.

Pereza-Echeverria, S., Herra-Valencia, V. A. and James, A. (2001). Detection of DNA methylation changes in micropropagated banana plants using methylation-sensitive amplification polymorphism (MSAP). *Plant Sci.***161**: 359-367.

Perez-Parron, M. A., Gonzalez-Benito, M. E. and Perez, C. (1994). Micropropagation of *Fraxinus angustifolia* from mature and juvenile plant material. *Plant Cell. Tiss. Org. Cult.* **37**: 297 - 302.

Pevalek-Kozlina, B. (1991). Clonal propagation of common oak (*Quercus robur* L.). *Acta Hort.* **289**: 143-144.

Pharr, D. M., Stoop, J. M. H., Williamson, J. D., Feusi, M. E. S., Massel, M. O., Conkling, M. A. (1995). The dual role of mannitol as osmoprotectant and photoassimilate in celery. *HortScience* **30**(6): 1182-1188.

Phillips, R. L., Kaeppler, S. M. and Olhoft, P. (1994). Genetic instability of plant tissue cultures: breakdown of normal controls. *P Natl. Acad. Sci. USA* **91**: 5222-5226.

Pierik, R. M. (1975). Plantenteelt in Kweekbuizen. Thieme, Zutphen, the Netherlands.

Pierik, R. M. (1979). *In vitro* culture of higher plants. Bibliography. Ponsen en Looyen, Wageningen, the Netherlands.

Pierik, R. M. (1987a). *In vitro* culture of higher plants. Dordecht, Martinus Nijhoff.

Pierik, R. M. (1987b). Preparation and composition of nutrient media. R. L. M. Pierik. *In vitro Culture of Higher Plants.* Dordrecht, Martinus Nijhoff: pp 45-85.

Pierik, R. M. (1990). Rejuvenation and micropropagation. H. J. Nijkamp, L. H. van der Plas and J. van Aartrijk. *Progress in plant cellular and molecular biology.* Dordrecht, The Netherlands, Kluwer Academic Publishers: 91-101.

Pierik, R. M. and Sprenkels, P. A. (1997). Micropropagation of *Fraxinus excelsior* L. (Common Ash). Y. P. S. Bajaj. *Biotechnology in Agriculture and Forestry.* Berlin Heidelberg, Springer-Verlag. **39:** 330 - 343.

Pilate, G., Sossountzov, L. and Miginiac, E. (1989). Hormone levels and apical dominance in the aquatic fern *Marsilea drummondii* A. Br. *Plant Physiol.* **90**(3): 907-912.

Pineda-Krch, M. and Lehtila, K. (2004). Costs and benefits of genetic heterogeneity within organisms. *J. Evol. Biol.* Doi:10.1111/j.1420-9101.2004.00808.x.

Piola, F., Rohr, R. and Heizmann, P. (1999). Rapid detection of genetic variation within and among *in vitro* propagated cedar (*Cedrus libani* Loudon) clones. *Plant Sci.* **141**: 159-163.

Piqueras, A., Cortina, M., Serna, M. D. and Casas, J. L. (2002). Polyamines and hyperhydricity in micropropagated carnation plants. *Plant Sci.* **162**: 671-678.

Pittet, H. and Moncousin, C. (1982). La micropropagation *in vitro* du rosier. *Rev. Hort. Suisse* **55**: 67-69.

Plant Cell Technology, I. (1998). PPM: A powerful technology to prevent and eliminate microbial contamination in plant tissue culture. http://mktechnology.com/ppmweb2.htm#what (8 May 1998).

Polanco, C. and Ruiz, M. L. (2002). AFLP analysis of somaclonal variation in *Arabidopsis thialiana* regenerated plants. *Plant Sci.* **162**(5): 817-824.

Poncia, G. and Roselli, G. (1980). Prove di micropropagazionedi due cloni di ciliegio acido (*Prunus cerasus* L.). *Rivista Ortofl. It.* **64**: 229-240.

Popovic, R. K., Kyle, D. J., Cohen, A. S. and Zalik, S. (1979). Stabilisation of thylakoid membranes by spermine during stress induced senescence of barley leaf discs. *Plant Physiol.* **64**: 721-726.

Powell, L. E. (1987). Hormonal aspects of bud and seed dormancy in temperate-zone woody plants. *HortScience* **22**: 845-850.

Preece, J. (1997). Axillary shoot proliferation. R. Geneve, J. Preece and S. Merkle. *Biotechnolgy of Ornamental Plants*, CAB International: 35-43.

Preece, J. and Sutter, E. (1991). Acclimatization of micropropagated plants to the greenhouse and field. P. Debergh and R. Zimmerman. *Micropropagation. Technology and Application.* Dordrecht, Kluwer: 71-93.

Preece, J. E. (1995). Can nutrient salts partially substitute for plant growth regulators? *Plant Tiss. Cult. Biotech.* **1**: 26-37.

Preece, J. E., Christ, P. H., Ensenberger, L. and Zhao, J. L. (1987). Micropropagation of ash (*Fraxinus*). *Comb. Proc. Int. Plant. Prop. Soc.* **37**: 366 - 372.

Preece, J. E. and Imel, M. R. (1991). Plant regeneration from leaf explants of Rhododendron "P.J.M. Hybrids". *Scientia Hort.* **48**: 159-170.

Preece, J. E., Zhao, J. and Kung, F. H. (1989). Callus production and somatic embryogenesis from white ash. *HortScience* **24**: 377-380.

Priebe, A., Klein, H. and Jager, A. J. (1978). Role of polyamines in SO2- polluted tea plants. *J. Exp. Bot.* **29**: 1045-1050.

Primmer, C. R. e. a. (1996). Directional evolution in germline microsatellite mutations. *Nat. Genet.* **13**: 391-393.

Primmer, C. R. e. a. (1998). Unravelling the process of microsatellite evolution through analysis of germ line mutations in barn swallows *Hirundo rustica*. *Mol. Biol. Evol.* **15**: 1047-1054.

Proskauer, J. and Berman, R. (1970). Agar culture medium modified to approximate soil conditions. *Nature* **227**: 1161.

Provan, J., Soranzo, N., Wilson, N. J., Goldstein, D. B. and Powell, W. (1999). A low mutation rate for chloroplast microsatellites. *Genetics* **153**: 943-947.

Puchta, H., Swoboda, P. and Hohn, B. (1995). Induction of intrachromosomal homologous recombination in whole plants. *Plant J.* **7**: 203-210.

Puente, J. and Marín, J. (1997). *In vitro* rootability of clonal apple microcuttings, derived from rooted and unrooted shoots. *Scientia Hort.* **68**: 227-230.

Quoirin, M. and Lepoivre, P. (1977). Ètude de milieux adaptès aux cultures *in vitro* de *Prunus*. *Acta Hort.* **78**: 437-442.

Quraishi, A., Koche, V., Sharma, P. and Mishra, S. K. (2004). *In vitro* Clonal Propagation of Neem (*Azadirachta indica*). *Plant Cell. Tiss. Org. Cult.* **78**: 281-284.

Rahman, M. H. and Rajora, O. P. (2001). Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). *Plant Cell Rep.* **20**(6): 531-536.

Rajam, M. V. (1997). Polyamines. M. N. V. Prasad. *Plant Ecophysiology*. New York, Wiley: pp 343-374.

Ramarosandratana, A., Harvengt, L., Bouvet, A., Galvayrac, R. and Paques, M. (2001). The effects of carbohydrate source, polyethylene glycol and gellan gum concentration on embryonal-suspensor mass (ESM) proliferation and maturation of maritime pine somatic embryos. *In Vitro Cell. Devel. Biol.- Plant* **37**: 29-34.

Raquin, C., Brachet, S., Jeandroz, S., Vedel, F. and Frascaria-Lacoste, N. (2002). Combined analysis of microsatellite and RAPD markers demonstrate possible hybridisation between *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl. *For. Gen.* **9**: 111-114.

Redenbaugh, K., Fuji, I. A. A. and Slade, D. (1993). Hydrated coatings for synthetic seed. K. Redenbaugh. *Synseed: Application of synthetic seeds to crop improvement.* Boca Raton (FL, USA), CRC Press Inc.: 35 - 46.

Rey, M., Tiburcio, A. F., Diaz-Sala, C. and Rodriguez, R. (1994). Endogenous polyamine concentrations in juvenile and adult and in vitro reinvigorated hazel. *Tree Physiol.* **14**: 191-200.

Richardson, S. (1958). Bud dormancy and root development in *Acer saccharinum.* K. Thinmann. *The Physiology of Forest Trees.* New York, Ronald Press,: 409-425.

Rieseberg, L. H. (1996). Homology among RAPD fragments in interspecific comparisons. *Mol. Ecol.* **5**: 99-105.

Righetti, B., Magnanini, E., Infante, R. and Predieri, S. (1990). Ethylene, ethanol, acetaldehyde and carbon dioxide released by *Prunus avium* shoot cultures. *Physiol. Plant.* **78**: 507-510.

Rinkevich, B. (2000). A critical approach to the definition of Darwinian units of selection. *Biol. Bull.* **199**: 231-240.

Rinkevich, B. and Shapira, M. (1999). Multi-partner urochordate chimeras outperform two-partner chimerical entities. *Oikos* **87**: 315-320.

Rinne, P. L. H., Kaikuranta, P. M. and van der Schoot, C. (2001). The shoot apical meristem restores its symplasmic organization during chilling-induced release from dormancy. *Plant J.* **26**: 249–264.

Riou-Khamlichi, C. (2000). Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. *Mol. Cell Biol.* **20**: 4513–4521.

Rival, A., Bertrans, L., Beule, T., Combes, M. C., Trouslot, P. and Lasheremes, P. (1998). Suitability of RAPD analysis for the detection of somaclonal variants in oil palm (*Elaeis guineensis* Jacq.). *Plant Breed.* **117**: 73-76.

Romberger, J. A. (1963). Meristems, growth and development in woody plants. *Technical Bulletin No. 1293*. Washington, DC, US Department of Agriculture.

Rosin, F. M., Hart, J. K., Horner, H. T., Davies, P. J. and Hannapel, D. J. (2003). Overexpression of a KNOTTED-like homeobox gene of potato alters vegetative development by decreasing gibberellin accumulation. *Plant Physiol.* **132**: 106–117.

Rowland, L. J. and Arora, R. (1997). Proteins related to endodormancy (rest) in woody perennials. *Plant Sci.* **126**: 119-144.

Rowland, L. J., Liu, D., Millard, M. M. and Line, M. J. (1992). Magnetic resonance imaging of water in flower buds of blueberry. *HortScience* **27**: 339-341.

Roy, S. K., Rahman, S. L. and Majumdar, R. (1990). *In vitro* propagation of jackfruit (*Artocarpus heterophyllus* Lam.). *J. Hort. Sci.* **65**: 355-358.

Ruaud, J. N., Bercetche, J. and Paques, M. (1992). First evidence of somatic embryogenesis from needles of 1 year old *Picea abies* plants. *Plant Cell Rep.* **11**: 563 - 566.

Ruigini, E. (1984). *In vitro* propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability and medium development using analytical data from developing shoot and embryos. *Scientia Hort.* **24**: 123-134.

Ruigini, E. (1986). Olive (*Olea europaea* L.). Y. P. S. Bajaj. *Biotechnology in agriculture and forestry. Trees I.* Berlin, Springer-Verlag. **1:** 253-267.

Ruigini, E., Pellegrineschi, A., Mencuccini, M., Mariotti, D. (1991). Increase of rooting ability in the woody species kiwi (*Actinidia deliciosa* A. Chev.) by transformation with *Agrobacterium rhizogenes rol* genes. *Plant Cell Rep.* **10**: 291-295.

Ruigini, E., Jacoboni, A. and Luppino, M. (1993). Role of basal shoot darkening and exogenous putrescine treatments on *in vitro* rooting and on endogenous polyamine changes in difficult to root woody species. *Scientia Hort.* **53**: 63-72.

Ruigini, E. and Verma, D. (1983). Micropropagation of difficult to propagate almond (*P. amygdalus* Batch.) cultivar. *Plant Sci. Lett.* **28**: 273-281.

Ruiz, M. L., Rueda, J., Pelaez, M. I., Espino, F. J., Candela, M., Sendino, A. M. and Vazquez, A. M. (1992). Somatic embryogenesis, plant regeneration and somaclonal variation in barley. *Plant Cell. Tiss. Org. Cult.* **28**: 97-101.

Sachs, S. (1880). Cited in: Audus L. (1959) *Plant Growth Substances*. 2nd Edition. Leonard Hill, London.

Sahijram, L. and Rajasekharan, P. E. (1997). Tissue Culture Strategies Applicable to *in vitro* Conservation of Tropical Fruit Crops. IPGRI - ICAR - UTANET Regional Training Course on the Conservation and Use of Germplasm of Tropical Fruits in Asia, Indian Institute of Horticultural Research, IPGRI.

Sahrawat, A. K., Suresh, C. and Chand, S. (1999). Stimulatory effect of copper on plant regeneration in indica rice (*Oryza sativa* L.). *J. Plant Physiol.* **154**: 517-522.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. And Evol.* **4**: 406-425.

Saker, M. M., Bekheet, S. A., Taha, H. S., Fahmy, A. S. and Moursy, H. A. (2000). Detection of somaclonal variation in tissue culture-derived date palm plants using isoenzyme analysis and RAPD fingerprints. *Biol. Plant.* **43**: 347-351.

Samish, R. M. (1954). Dormancy in woody plants. *Ann. Rev. Plant Physiol.* **5**: 183-200.

Sánchez, M. and Vieitez, A. (1991). *In vitro* morphogenetic competence of basal sprouts and crown branches of mature chestnut. *Tree Physiol.* **8**: 59-70.

Sánchez, M. C., San-Jose, M. C., Ballester, A. and Vieitez, A. M. (1996). Requirements for *in vitro* rooting of *Quercus robur* and *Q. rubra* shoots derived from mature trees. *Tree Physiol.* **16**: 673-680.

San-José, M., Ballester, A. and Vieitiz, A. (1988). Factors affecting *in vitro* propagation of *Quercus robur* L. *Tree Physiol.* **4**: 281-290.

San-Jose, M. C., Vietiez, A. M. and Ballester, A. (1990). Clonal propagation of Juvenile and Adult trees of sessile oak by tissue culture techniques. *Sil. Gen.* **32**(2): 50-55.

Santelices, B. (1999). How many kinds of individual are there? *Trends Ecol. Evol.* **14**: 152-155.

Santelices, B. (2004). Mosaicism and chimerism as components of intraorganismal genetic heterogeneity. *J. Evol. Biol.* Doi:10.1111/j.1420-9101.2004.00813.x.

Santos, C. V., Brito, G., Pinto, G. and Fonseca, M. A. C. (2003). *In vitro* plantlet regeration of *Olea europaea* ssp. *maderensis. Scientia Hort.* **97**: 83-87.

Satina, S. and Blakeslee, A. F. (1941). Periclinal chimeras in *Datura stramonium* in relation to development of leaf and flower. *Am. J. Bot.* ***28***: 862-871.

Satina, S., Blakeslee, A. F. and Avery, A. G. (1940). Demonstration of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. *Am. J. Bot.* ***27***: 895-905.

Saure, M. (1985). Dormancy release in deciduous fruit trees. J. Janick. *Horticultural Reviews Vol 7.* Westport, Connecticut, AVI Publishing: 239-300.

Sauter, M. (1997). Differential expression of a *CAK* (*cdc2*-activating kinase)-like protein kinase, cyclins and *cdc2* genes from rice during the cell cycle and in response to gibberellin. *Plant J.* 11(2): 181-190.

Scalbert, A., Monties, B. and Favre, J.-M. (1988). Polyphenols of *Quercus robur*: Adult tree and *in vitro* grown calli and shoots. *Phytochemistry* **27**: 3483-3488.

Schaal, B. A. (1987). Somatic variation and genetic structure in plant populations. A. J. Davy, M. J. Hutchings and A. R. Watkinson. *Plant Population Ecology*. Boston, Blackwell Scientific Publications: 47-58.

Schaal, B. A. and Learn, G. H. (1988). Ribosomal DNA variation within and among plant populations. *Ann. Mol. Bot. Gard.* **75**: 1207- 1216.

Schlötterer, C. and Tautz, D. (1992). Slippage synthesis of simple sequence DNA. *Nucleic Acids Res.* **20**: 211-215.

Schlötterer, C. R. (2000). Evolutionary dynamics of microsatellite DNA. *Chromosoma* **109**: 365-371.

Schlötterer, C. R., Ritter, R., Harr, B. and Brem, G. (1998). High mutation rate of a long microsatellite allele in *Drosophila melanogaster* provides evidence for allele-specific mutation rates. *Mol. Biol. Evol.* **15**: 1269-1274.

Schmidt, G. (1995). Methods for difficult hardwood cuttings with bottom heat in Hungary. *Comb. Proc. Int. Plant Prop. Soc.* **45**: 161-164.

Schnapp, S. R. and Preece, J. E. (1986). *In vitro* growth reduction of tomato and carnation microplants. *Plant Cell. Tiss. Org. Cult.***6**: 3-8.

Schwann, T. H. (1839). Mikroskepische Untersuchungen uber die Ubereinstimmung in der Tiere und Pflanzen. Oswalds Klassiker der exakten Wissenshaften, 1910.

Scott, P., Lyne, R. L. and Rees, T. (1995). Metabolism of maltose and sucrose by microspores isolated from barley (*Hordeum vulgare*). *Planta* **197**: 435-441.

Selby, C. and Harvey, B. M. R. (1985). The influence of natural and *in vitro* bud flushing on adventitious bud production in sitka spruce (*Picea sitchensis* (Bong.) Carr.) bud and needle cultures. *New Phytologist.* **100**: 549-562.

Sensi, E., Vignani, R., Scali, M., Masi, E. and Cresti, M. (2003). DNA fingerprinting and genetic relatedness among cultivated varieties of *Olea europaea* L. estimated by AFLP analysis. *Scientia Hort.* **97**: 379-388.

Sharma , P. and Rajam, M. V. (1995). Spatial and temporal changes in endogenous polyamine levels associated with somatic embryogenesis from different hypocotyl segments of eggplant (*Solanum melongena* L.). *J. Plant Physiol.* **146**: 658-664.

Shelp, B., Walton, C., Snedden, W., Tuin, L., Oresnik, I. and Layzell, D. (1995). GABA-shunt in developing soy-bean is associated with hypoxia. *Physiol. Plant.* **94**: 219-228.

Shen, H. J., Xie, Y. F. and Li, R. T. (1994). Effects of acid stress on polyamine levels, ion efflux, protective enzymes and macro-molecular synthesis in cereal leaves. *Plant Growth Reg.* **14**:1-5.

Shen, W.-H. (2002). The plant E2F–Rb pathway and epigenetic control. *Trends Plant Sci.* **11**: 505 – 511.

Shirazi, A. M. and Fuchigami, L. H. (1993). Recovery of plants from "near-lethal" stress. *Oecologia* **93**: 429--434.

Shirazi, A. M. and Fuchigami, L. H. (1995). Effects of "near lethal" stress on bud dormancy and stem cold hardiness in red-osier dogwood. *Tree Physiol.* **15**: 275 - 279.

Shirazi, A. M., Siller-Cepeda, J. H. and Fuchigame, L. H. (1994). Role of glutathione in recovery of red-osier dogwood plants from near-lethal heat stress. *Can. J. For. Res.* **24**: 415-418.

Shoeb, F., Yadav, J. S., Bajaj, S. and Rajam, M. V. (2001). Polyamines as biomarkers for plan regeneration capacity: improvement of regeneration by modulation of polyamine metabolism in different genotypes of indica rice. *Plant Sci.* **160**: 1229-1235.

Shoemaker, R. C., Amberger, L. A., Palmer, R. G., Oglesby, L. and Ranch, J. P. (1991). The effect of 2,4-dichlorophenoxyacetic acid concentration on somatic embryogenesis and heritable variation in soybean (*Glycine max* (L.) Merr.). *In Vitro Cell. Dev. Biol. - Plant* **27**: 84-88.

Shriver, M. D., Jin, L., Chakraborty, R. and Boerwinkle, E. (1993). VNTR allele frequency distribution under the stepwise mutation model: A computer simulation approach. *Genetics* **134**: 983-993.

Shulman, Y., Nir, G., Fanberstein, L. and Lavee, S. (1983). The effect of cyanamide on the release from dormancy of grapevine buds. *Scientia Hort.* **19**: 97-104.

Silander, J. A. (1985). Microevolution in clonal plants. J. Jackson, L. Buss and R. Cook. *Population Biology and Evolution of Clonal Organisms*. New Haven, CT, Yale University Press: 107-152.

Siller-Cepeda, J. H., Fuchigami, L. H. and Chen, T. H. H. (1992). Glutathione content in peach buds in relation to developement and release of rest. *Plant Cell Physiol.* **33**: 867 - 872.

Silveira, C. E. and Cottignies, A. (1994). Period of harvest, sprouting ability of cuttings, and *in vitro* plant regeneration in *Fraxinus excelsior*. *Can. J. Bot.* **72**: 261-267.

Sitbon, F. and Perrot-Recchenmann, C. (1997). Expression of auxin-regulated genes. *Physiol. Plant.***100**: 443-455.

Sivanesan, A. and Waller, J. M. (1986). Sugarcane diseases. CMI Phytopathology Paper No.29. Wallingford, UK., CAB International.

Skinner, E. (1964). Delayed foliation. *Decid. Fruit Grower* **14**: 195-197.

Skirvin, R. M., Kouider, M., Joung, H. and Korban, S. S. (1986). Apple (*Malus* x *domestica* Borkh.). Y. P. S. Bajaj. *Biotechnology in Agriculture and Forestry. Trees 1*. Berlin, Springer-Verlag. **1:** 183-198.

Skirvin, R. M., McPheeters, K. D. and Norton, M. (1994). Sources and frequencies of somaclonal variation. *HortScience* **29**: 1232-1237.

Skoog, F. and Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp. Soc. Exp. Biol.* **11**: 118 - 130.

Slatkin, M. and Barton, N. H. (1989). A comparison of three methods for estimating average levels of gene flow. *Evolution* **43**: 1349-1368.

Slocum, R. D., Kuar-Sawheny, R. and Galston, A. W. (1984). The physiology and biochemistry of polyamines in plants. *Arch. Biochem. Biophys.* **235**: 283-303.

Smith, D. and Thorpe, T. (1975). Root initiation in cuttings of *Pinus radiata* seedlings. 1. Developmental sequence. *J. Exp. Bot.* **26**: 184-192.

Smith, D. N. and Devey, M. E. (1994). Occurrence and inheritance of microsatellites in *Pinus radiata*. *Genome* **37**: 977-983.

Smith, T. A. (1985). Polyamines. *Ann. Rev. Plant Physiol.* **36**: 117-143.

Smulders, M. J. M., van der Schoot, J., Arens, P. and Vosman, B. (2001). Trinucleotide repeat microsatellite markers for Black Poplar (*Populus nigra* L.). *Mol. Ecol. Notes* **1**: 188.

Sneath, P. H. A. and Sokal, R. R. (1973). Numerical Taxonomy. San Francisco, W. H. Freeman and Company.

Soneji, J. R., Rao, P. S. and Mhatre, M. (2002). Somaclonal variation in micropropagated dormant axillary buds of pineapple (*Ananas comosus* L., Merr.). *J. Hortic. Sci. Biotech.* **77**: 28-32.

Spena, A., Schmulling, T., Konca, C., Schell, J. (1987). Independent and synergistic activity of *rol A, B* and *C* loci in stimulating abnormal growth in plants. *The Embo Journal* **6**: 3891-3899.

Sriskandarajah, S., Mullins, M. G. and Nair, Y. (1982). Induction of adventitious rooting *in vitro* with difficult to propagate cultivars of apple. *Plant Sci. Lett.* **24**: 1 - 9.

Srivastava, L. M. (2002). Plant Growth and Development: *Hormones and Environment*. Amsterdam, Academic Press.

Srivastava, P. S. and Steinhauer, A. (1982). *In vitro* culture of embryo segments of *Quercus lebani*: Organogenesis and callus growth as a differential response to experimental conditions. *Z. Pflanzenphysiol.* **106**: 93-96.

Stals, H. and Inzè, D. (2001). When plant cells decide to divide. *Trends Plant Sci.* **6**: 359–364.

StatSoft, I. (2001). STATISTICA for Windows [Computer program manual]. Tulsa, OK, U. S. A., StatSoft, Inc.

Stoler, D. L., Cheng, N., Basik, M., Kahlenberg, M. S., Rodriguez-Bigas, M. A., Petrelli, N. J. and Anderson, G. R. (1999). The onset and extent of genomic instability in sporadic colorectal tumour progression. *Proc. Natl. Acad. Sci. U.S.A.* **96**: 15121-15126.

Stoop, J. M. H., Williamson, J. D., Pharr, D. M. (1996). Mannitol metabolism in plants: a method for coping with stress. *Trends Plant Sci.* **1**: 139-144.

Strand, M., Prolia, T. A., Liskay, R. M. and Petes, T. D. (1993). Destabilisation of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* **365**: 274-276.

Street, H. E. (1973). Plant tissue and cell culture. Oxford, Blakwell Scientific Publishers.

Streiff, e. a. (1999). Pollen dispersal inferred from paternity analysis in a mixed oak stand of *Quercus robur* L. and *Q. petraea* (Matt.) Liebl. *Mol. Ecol.* **8**: 831-841.

Suomela, J. and Nilson, A. (1994). Within-tree and among-tree variation in growth of *Epirrita autumnata* on mountain birch leaves. *Ecol. Entomol.* **19**: 45-56.

Sutter, E. and Langhans, R. W. (1979). Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. *J. Amer. Soc. Hort. Sci.* **104**: 493 - 496.

Sutter, E. and Langhans, R. W. (1980). Formation of epicuticular wax and its effect on water loss in cabbage plants regenerated from shoot tip culture. *HortScience* **15**: 428 - 429.

Sutter, E. and Luza, J. (1993). Developmental anatomy of roots induced by *Agrobacterium rhizogenes* in *Malus pumila* 'M26' shoots grown *in vitro*. *Int. J. Plant Sci.* **154**: 59-67.

Suttle, J. C. (1998). Involvement of ethylene in potato microtuber dormancy. *Plant Physiol.***118**: 843–848.

Swofford, D. L. and Begle, D. P. (1993). PAUP Phylogenetic Analysis Using Parsimony. Version 3.1. User's Manual., Illinois Natural History Survey: pp. 1-57.

Taberlet, P., Friffin, S., Goossens, B., Questiau, S., Manceau, V. and et al. (1996). Reliable genotyping of samples with very low DNA quantities. *Nucleic Acids Res.* **24**: 3189-3194.

Tabor, C. W. and Tabor, H. (1984). Polyamines. *Ann. Rev. Biochem.* **53**: 749-790.

Tabrett, A. M. and Hammatt, N. (1992). Regeneration of shoots from embryo hypocotyls of common ash (*Fraxinus excelsior* L.). *Plant Cell Rep.* **11**: 514 - 518.

Tamas, I. A. (1995). Hormonal regulation of apical dominance. P. J. Davies. *Plant hormones-physiology, biochemistry and molecular biology.* Netherlands, Kluwer Academic: 572-597.

Taylor, B. and Scheuring, C. (1994). A molecular marker for lateral root initiation: The RSI-1 gene of tomato (*Lycopersicon esculentum* Mill) is activated in early lateral root primordia. *Mol. Gen. Genet.* **243**: 148-157.

Taylor, M. B. and Henshaw, G. G. (1990). The production of potato chimeras through *in vitro* grafting. *Abstracts of the 7th international congress of plant tissue and cell culture. Poster A5-41. Amsterdam.*

Thibodeau, S. N., Bren, G. and D., S. (1993). Microsatellite instability in cancer of the proximal colon. *Science* **260**: 816-819.

Thom, T. A., Maretzki, E. and Sakai, H. S. (1981). Nutrient uptake and accumulation by sugarcane cell culture in relation to growth cycle. *Plant Cell. Tiss. Org. Cult.* **1**: 3-14.

Thomas, G. (1986). Geneflow. Rome, International Board for Plant Genetic Resources.

Thompson, G. A. (1979). Molecular control of membrane fluidity. J. M. Lyons, D. Grahas and J. K. Raison. *Low Temperatire Stress in Crop Plants.* New York, Academic Press: pp 347-363.

Thompson, J. E., Legge, R. L. and Barber, R. F. (1987). The role of free radicals in senescence and wounding. *New Phytologist.* **105**: 317 - 344.

Thomson, J. D., Herre, E. A., Hamrick, J. L. and Stone, J. L. (1991). Genetic mosaics in strangler fig trees: implications for tropical conservation. *Science* **254**: 1214-1216.

Thorpe, T. A. and Biondi, S. (1984). Conifers. W. R. Sharpe, D. A. Evans, P. V. Ammirato and Y. Yamada. *Handbook of Plant Cell Culture*. New York, Macmillan. **2:** 435 - 470.

Thorpe, T. A., Harry, I. S. and Kumar, P. P. (1991). Application of micropropagation to forestry. P. C. Debergh and R. H. Zimmerman. *Micropropagation: Technology and Application.* Netherlands, Kluwer Academic Publishers: 311 - 336.

Tiburcio, A., Gendy, C. and Tran Tanh Van, K. (1989). Morphogenesis in tobacco subepidermal cells: putrescine as a marker of root differentiation. *Plant Cell. Tiss. Org. Cult.***19**: 43-54.

Tiburcio, A. F., Campos, J. L., Figueras, X. and Besford, R. T. (1993). Recent advances in the understanding of polyamine functions during plant development. *Plant Growth Reg.* **12**: 331-340.

Tiburcio, A. F., Kaur-Sawhey, R. and Galston, A. W. (1990). Polyamine metabolism. B. J. Miflin and P. J. Lea. *The Biochemistry of Plants, Intermediary Nitrogen Fixation.* New York, Academic: pp 283 - 325.

Tiburcio, A. F., Kuar-Sawhney, R. and Galston, A. W. (1987). Polyamines and organogenesis in thin layer tobacco tissue cultures. M. B. Jackson, S. H. Mantell and J. Blake. *Advances in the Chemical Manipulation of Plant Tissue Cultures.* Bristol, Monograph 16, British Plant Growth Regulator Group: 43-56.

Tilney-Bassett, R. A. (1986.). Plant Chimeras. London, Edward Arnold.

Timber Research and Development Association (1980). Timbers of the World. England, The Construction Press.

Tonon, G., Capuana, M. and Di Marco, A. (2001a). Plant regeneration of *Fraxinus angustifolia* by *in vitro* shoot organogenesis. *Scientia Hort.* **87**: 291-301.

Tonon, G., Capuana, M. and Rossi, C. (2001b). Somatic embryogenesis and enbryo encapsulation in *Fraxinus angustifolia* Vhal. *J. Hortic. Sci. Biotech.* **76**: 753-757.

Tonon, G., Kevers, C., Faivre-Rampant, O., Graziani, M. and Gaspar, T. (2004). Effect of NaCl and mannitol iso-osmotic stresses on proline and free polyamine levels in embryogenic *Fraxinus angustifolia* callus. *J. Plant Physiol.* **161**: 701-708.

Torrey, J. (1963). Cellular patterns in developing roots. *Symp. Soc. Exp. Biol.* **17**: 285-314.

Torrey, J. G. (1956). Chemical factors limiting lateral root formation in isolated pea roots. *Physiol. Plant.* **9**: 370- 388.

Torrigiani, P., Altamura, M., Capitani, F., Falasca, G., Bagni, N. and Serafini-Fracassini, D. (1990). Inhibition of polyamine biosynthesis affects the rhizogenic response in tobacco thin cell layers. H. Nijkamp, L. Van Der Plas and V. Aartrijk. *Progress in Plant Cellular and Molecular Biology. Proceedings of the VIIth International Congress on Plant Tissue & Cell Culture. Amsterdam, The Netherlands. 24-29 June 1990.* Dortrecht, Netherlands, Kluwer Academic Publishers: 480-485.

Travis, S. E., Maschinski, J. and Keim, P. (1996). An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. *Mol. Ecol.* **5**: 735-745.

Trip, P., Krotkov, G. and Nelson, C. D. (1964). Metabolism of mannitol in higher plants. *Am. J. Bot.* **51**: 828-835.

Tsumara, Y., Ohba, K. and Strauss, S. H. (1996). Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir *(Pseudotsuga menziesii)* and sugi *(Cryptomeria ja-ponica)*. *Theor. Appl. Genet.* **92**: 40-45.

Tuomi, J. and Vuorisalo, T. (1989). Hierarchical selection in modular organisms. *Trends Ecol. Evol.* **4**: 209-213.

Tuskan, G. A., Francis, K. E., Russ, S. L., Romme, W. H. and Turner, M. G. (1996). RAPD markers reveal diversity within and among clonal and seedling stands of aspen in Yellowstone National Park, USA. *Can. J. For. Res.* **26**: 2088-2098.

Udupa, S. M. and Baum, M. (2001). High mutation rate and mutational bias at (TAA)n loci in chickpea (*Cicer arietinum* L). *Mol. Gen. Genet.* **265**: 1097-1103.

Valdes, A. M., Slatkin, M. and Freimer, N. B. (1993). Allele frequencies at microsatellite loci: The stepwise mutation model revisited. *Genetics* **133**: 737-749.

van der Lek, H. (1925). Root development in woody cuttings. *Med Landbouwhogescholl Wageningen* **28**: 211-230.

Van Huylenbroeck , J. and De Riek, J. (1995). Sugar and starch metabolism during ex vitro rooting and acclimatization of micropropagated *Spathiphyllum* 'Petite' plantlets. *Plant Sci.* **111**: 19-25.

Van Huylenbroeck, J., Huygens, H. and Debergh, P. (1995). Photoinhibition during acclimitization of micropropagated *Spathiphyllum* 'Petite' plantlets. *In Vitro Cell. Dev. Biol.* **31**: 160-164.

Van Nieuwkerk, J. P., Zimmerman, R. H. and Fordham, I. (1986). Thidiazuron stimulation of apple shoot proliferation *in vitro*. *HortScience* **21**: 516-518.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P., and Shipley, P. (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**: 535-538.

Varga , M. and Humphries, E. (1974). Root formation on petioles of detached primary leaves of dwarf bean (*Phaseolus vulgaris*) pretreated with gibberellic acid, triiodobenzoic acid, and cytokinins. *Ann. Bot.* **38**: 803-807.

Vasil, I. K., Ahuja, M. R. and Vasil, V. (1979). Plant tissue culture in genetics and plant breeding. *Adv. Genet.* **20**: 127-215.

Vasil, I. K. and Vasil, V. (1980). Clonal propagation. I. K. Vasil. *Perspectives in Plant Cell and Tissue Culture*. New York, Academic Press: 145-173.

Vegis, A. (1932). Uber das Fruhtreibender Winterknospen von *Hydrocharis morusus ranael* durchy hochtemperierte Wasserbader. *Jahrb. F. Wiss. Bot.* **75**: 726-770.

Vegis, A. (1964). Dormancy in higher plants. *Ann. Rev. Plant Physiol.* **15**: 185-224.

Vendrame, W. A., Kochert, G. D., Sparks, D. and Wetzstein, H. Y. (2000). Field performance and molecular evaluations of pecan trees regenerated from somatic embryogenic cultures. *J. Amer. Soc. Hort. Sci.* **125**: 542-546.

Vendrame, W. A., Kochert, G. D. and Wetzstein, H. Y. (1999). AFLP analysis of variation in pecan somatic embryos. *Plant Cell Rep.* **18**: 853-857.

Vengadesan, G., Ganapathi, A., Amutha, S. and Selvaraj, N. (2002). *In vitro* propagation of *Acacia* species - a review. *Plant Sci.* **163**: 663-671.

Vera, P., Lamb, C. and Doerner, P. (1994). Cell cycle regulation of hydroxyproline-rich glycoprotein HRGPnt3 gene experssion during the initiation of lateral root meristems. *Plant J.* **6**: 717-727.

Vieitez, A. M., San-Jose, M. C. and Vieitez, E. (1985). *In vitro* plantlet regeneration from juvenile and mature *Quercus robur* L. *J. Hortic. Sci.* **60**: 99-106.

Vieititez, A. M., Vieititez, M. L. and Vieititez, E. (1986). Chestnut (Castanea spp.). Y. P. S. Bajaj. *Biotechnology in Agriculture and Forestry. Trees 1.* Berlin, Springer-Verlag. **1:** 393-414.

Villalobos, V. M., Oliver, M. J., Yeung, E. C. and Thorpe, T. A. (1984) Cytokinin-induced switch in development in excised cotyledons of radiate pine cultured *in vitro*. *Physiol. Plant.* **61**: 483-489.

Vítová, L., Stodulkova, E., Bartonickova, A. and Lipavska, H. 2002. Mannitol utilisation by celery (*Apium graveolens*) plants grown under different conditions *in vitro*. *Plant Sci.* **163**: 907-916

Von Aderkas, P. and Bonga, J. M. (1998). Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree Physiol.* **20**: 921 - 928.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijtens, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**: 4407-4414.

Walter, H. J. P. and Geuns, J. M. C. (1987). High speed HPLC analysis of polyamines in plant tissues. *Plant Physiol.* **83**: 232-234.

Walters, D. R. (2003). Polyamines and plant disease. *Phytochemistry* **64**: 97 - 107.

Wandeler, P., Smith, S., Morin, P. A., Pettifor, R. A. and Funk, S. M. (2003). Patterns of nuclear DNA degradation over time- a case study in historic teeth samples. *Mol. Ecol.* **12**: 1087-1093.

Wang, S. W. and Faust, M. (1990). Changes in menbrane lipids in apple buds during dormancy and bud break. *J. Amer. Soc. Hort. Sci.* **115**: 803-808.

Wang, S. Y. and Faust, M. (1986). Effect of growth retardants on root formation and polyamine content in apple seedlings. *J. Amer. Soc. Hort. Sci.* **111**: 912-917.

Wang, S. Y. and Faust, M. (1988). Metabolic activities during dormancy and blomming of deciduous fruit tress. *Israel J. Bot.* **37**: 227-243.

Wang, S. Y. and Faust, M. (1994). Changes in the antioxidant system associated with bud break in 'Anna' apple (*Malus domestica* Borkh.) buds. *J. Amer. Soc. Hort. Sci.* **119**: 735--741.

Wanstreet, A. (1982). *In vitro* inoculation of tissue culture propagated *Malus* shoot tips with *Erwinia amylovora*. Columbus, OH, Ohio State University.

Wardle, P. (1961). Biological Flora of the British Isles, *Fraxinus excelsior* L. *J. Ecol.* **49**: 739 - 751.

Wattier, R., Engel, C. R., Saumitou-Laprade, P. and Valero, M. (1998). Short allele dominance as a source of heterozygote deficiency at microsatellite loci: experimental eveidence at the dinucleotide locus Gv1CT in *Gracilaria gracilis* (Rhodophyta). *Mol. Ecol.* **7**: 1569-1573.

Waxler, M. S. and van Buijten, J. P. (1981). Early genetic evaluation of Lobolly pine. *Can. J For. Res.* **11**: 351 - 355.

Weatherhead, M. A., Burdon, J. and Henshaw, G. G. (1978). Some effects of activated charcoal as an additive to plant tissue culture media. *Z. Pflanzenphysiol.* **89**: 141-147.

Weatherhead, M. A., Burdon, J. and Henshaw, G. G. (1979). Effects of activated charcoal as an additive to plant tissue culture media. *Z. Pflanzenphysiol.* **94**: 399-405.

Weatherwax, S. C., Williams, S. A., Tingay, S. and Tobin, E. M. (1998). The phytochrome response of the *Lemna gibba NPR1* gene is mediated primarily through changes in abscisic acid levels. *Plan Physiol.* **116**: 1299–1305.

Webb, D. A., Parnell, J. and Doogue, D. (1996). An Irish Flora. Dundalk, Dundalgan Press Ltd.

Weber, J. L. (1990). Informativeness of human (dC-dA)n and (dG-dT)n polymorphisms. *Genomics* **7**: 524-530.

Weber, J. L. and May, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* **44**: 388-396.

Weber, J. L. and Wong, C. (1993). Mutation of human short tandem repeats. *Hum Mol Genet.* **2**: 1123-1128.

Webster, C. A. and Jones, O. P. (1989). Micropropagation of the apple rootstock M. 9: effect of sustained subculture on apparent rejuvenation *in vitro*. *J. Hort. Sci.* **64**: 421 - 428.

Weigel, U., Horn, W. and Hock, B. (1984). Endogenous auxin levels in terminal stem cutting of *Chrysanthemum morifolium* during adventitious rooting. *Physiol. Plant.* **61**: 422-428.

Weisman, Z., Avidan, N., Lavee, S. and Quebedeaux, B. (1998). Molecular characterisation of common olive varietys in Israel and the West Bank using Randomly Amplified Polymorphic DNA (RAPD) Markers. *J. Amer. Soc. Hort. Sci.* **123**: 837-841.

Welander, M. (1983). *In vitro* rooting of the apple rootstocks M.26 in adult and juvenile growth phases and acclimatization of the plantlets. *Physiol. Plant.* **58**: 231-238.

Welander, M. (1988). Biochemical and anatomical studies of Birch (*Betula pendula* Roth.) buds exposed to different climatic conditions in relation to growth *in vitro*. J. W. Hanover and D. E. Keathley. *Genetic manipulation of woody plants*. Plenum Publishing Corporation.: 79-99.

Welch, H. (1970). Mist Propagation and Automatic Watering. London, Faber and Faber.

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**: 7213-7218.

Werner, M. (1996). Application of vegetative propagation to tree breeding. D. Thompson and M. Welander. *Molecular and Morphological Markers for Juvenility, Maturity, Rejuvenation and Somatic Embryogenesis in Woody Plant Species*. Malmo, Sweden, European Commision: 75-79.

White, J. and Lovell, P. (1984). Anatomical changes which occur in cuttings of *Agathis australis* (D. Don) Lindl. 2. The initiation of root primordia and early root development. *Ann. Bot.* **54**: 633-646.

White, P. R. (1943). A Handbook of Plant Tissue Culture. Lancaster, Pa., The Jaques Catlell Press.

White, P. R. (1954). The Cultivation of Animal and Plant Cells. New York, Ronald Press.

Whitham, T. G. and Slobodchikoff, C. N. (1981). Evolution by individuals, plant-herbivore interactions, and mosaics of genetic variability: the adaptive significance of somatic mutations in plants. *Oecologia* **49**: 287-292.

Wickson, M. and Thimann, K. V. (1958). The antagonism of auxin and kinetin in apical dominance. *Physiol. Plant.* **11**: 63 - 74.

Wilhelm, E. (2000). Somatic embryogenesis in oak (Quercus spp.). *In Vitro Cell Dev. Biol. Plant* **36**: 349-357.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6231-6235.

Winans, S. (1992). Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiological Reviews* **56**: 12-31.

Winkler, H. (1907). Uber Pfropfbastarde und pflanzliche Chimaren. *Berichte der Deutschen Botanischen Gesellschaft* **25**: 568-576.

Wisniewski, M., Fuchigami, L. H., Sauter, J. J., Shirazi, A. and Zhen, L. (1996). Sublethal stress and bud dormancy in woody plants. G. A. Lang. *Plant Dormancy: Physiology, Biochemistry, and Molecular Biology.* Oxon, U. K., CAB International: 201 - 210.

Wisniewski, M., Sauter, J., Fuchigami, L. and Stepien, V. (1997). Effects of near-lethal heat stress on bud break, heat shock proteins and ubiquitin in dormant poplar (*Populus nigra Charkowiensis* x *P. nigra incrassata*). *Tree Physiol.* **17**: 453 - 460.

Wisniewski, M., Sauter, J. J., Stepien, V. and Fuchigami, L. H. (1994). Effects of sublethal heat stress on endodormancy and ecodormancy of peach and hybrid poplar. *HortScience* **29**(5): 511.

Withers, L. A. and Engelmann, F. (1990). *In vitro* conservation of plant genetic resources. A. Altman. *Biotechnology in Agriculture*. New York, Marcel Dekker Inc.

Wolff, R. K., Plaetke, R., Jeffreys, A. J. and White, R. (1989). Unequal crossing over between homologous chromosomes is not the major mechanism involved in the generation of new alleles at VNTR loci. *Genomics* **5**: 382-384.

Woo, H., Hackett, W. and Das, A. (1994). Differential expression of a chlorophyll a/b binding protein gene in juvenile and mature phase English iv (*Hedera helix*). *Physiol. Plant.* **92**: 69-78.

Wood, H. G. and Kumar, G. K. (1985). Transcarboxylase – its quaternary structure and the role of the biotinyl subunit in the assembly of the enzyme and in catalysis. *Ann. NY Acad. Sci.* **447**: 1-22.

Woolley, D. J. and Wareing, P. F. (1972a). The role of roots, cytokinins and apical dominance in the control of lateral shoot form in *Solanum andigena*. *Planta* **105**: 33-42.

Woolley, D. J. and Wareing, P. F. (1972b). The interaction between growth promoters in apical dominance. II. Environmental effects on endogenous cytokinin and gibberellin levels in *Solanum andigena*. *New Phytologist* **71**: 1015-1025.

Wright, S. (1921). Systems of mating. *Genetics* **6**: 111-178.

Wright, S. (1969). Evolution and the genetics of populations. Vol. 2. The theory of gene frequencies. University of Chicago Press.

Yagishita, N. and Hirata, Y. (1987). Graft-induced change in fruit shape in *Capsicum annuum* L. I. Genetic analysis by crossing. *Euphytica* **36**: 809-814.

Yagishita, N., Hirata, Y., Mizukami, H., Ohashi, H. and Yamashita, K. (1990). Genetic nature of low capsaicin content in the variant strains induced by grafting in *Capsicum annuum* L. *Euphytica* **46**: 249-252.

Young, M. and Westwood, M. (1975). Influence of wounding and chilling on rooting of pear cuttings. *HortScience* **10**: 399-400.

Youssoufian, H. and Pyeritz, R. (2002). Mechanisms and consequences of somatic mosaicism in humans. *Nat. Rev. Genet.* **3**: 748-758.

Yu, K. and Pauls, K. P. (1994). Optimization of DNA-extraction and PCR procedures for Random Amplified Polymorphic DNA (RAPD) analysis in plants. H. G. Griffin and A. M. Griffin. *PCR Technology: Current Innovations.* CRC Press Inc: 193-203.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* **20**: 176-183.

Zimmerman, R. (1984). Rooting of apple cultivars *in vitro*: interactions among light, temperature, phloroglucinol and auxin. *Plant Cell. Tiss. Org. Cult.* **3**: 301-311.

Zimmerman, R. and Fordham, I. (1985). Simplified method for rooting apple cultivars *in vitro*. *J. Amer. Soc. Hort. Sci.* **110**: 34-38.

Ziv, M. (1991). Vitrification: morphological and physiological disorders of *in vitro* plants. P. C. Debergh and R. H. Zimmerman. *Micropropagation: technology and application.* Dordrecht, Kluwer Academic Publishers: pp 45-69.

Zobel, B. Z. and Talbert, J. T. (1984). Applied Forest Tree Improvement. New York, Wiley & Sons.

Zulini, L., Fabro, E. and Peterlunger, E. (2003). Genetic analysis of the grapevine cultivar 'Picolit' based on microsatellite and AFLP markers. *Acta Hort.* **603**: 467-472.

Appendix 1.

Nutrient composition of the culture media:

Media	*M91* (mg/l)*	*QRC (mg/l)*	*Modified WPM (mg/l)*
	MACROELEMENTS		
NH_4NO_3	1650	400	400
KNO_3	1900	-	-
$CaCl_2.2H_20$	440	96	96
$Ca(NO_3)_2.4H_2O$	-	556	556
$MgSO_4.7H_2O$	370	370	370
KH_2PO_4	170	-	170
K_2SO_4	-	990	990
KCl	-	-	-
	MICROELEMENTS		
KI	0.83	-	-
H_3BO_3	62	6.2	62
$MnSO_4.4H_2O$	223	22.3	29.43
$ZnSO_4.7H_2O$	86	8.6	86
$CuSO_4.5H_2O$	0.25	0.25	2.5
$Na_2MoO_4.2H_2O$	2.5	0.25	2.5
$CoCl_2.6H_2O$	0.25	-	-
$((NH_4)_2HPO_4)$	-	5.3	-
$NH_4H_2PO_4$	-	57.3	-
Sequestrene Fe 330	40	40	40
	VITAMINS		
Myo-Inistol	100	50	100
Nicotininc acid	1	-	0.5
Pyridoxine HCl	1	-	0.5
Thiamine	10	0.25	0.1
Glycine	-	-	2
Folic acid	-	-	-
Biotin	-	-	-

*Also referred to as modified MS medium type B.

Appendix 2.

Nutrient composition of the culture media:

Media	*Modified OM (mg/l)*	*DKW (mg/l)*
	MACROELEMENTS	
NH_4NO_3	412	1416
KNO_3	1100	-
$CaCl_2.2H_20$	332.2	112.5
$Ca(NO_3)_2.4H_2O$	416.9	1367
$MgSO_4.7H_2O$	732.6	361.5
KH_2PO_4	340	265
K_2SO_4	-	1559
KCl	500	-
	MICROELEMENTS	
KI	0.83	-
H_3BO_3	124	4.8
$MnSO_4.4H_2O$	169	33.8
$ZnSO_4.7H_2O$	143	-
$CuSO_4.5H_2O$	25	0.25
$Na_2MoO_4.2H_2O$	25	0.39
$CoCl_2.6H_2O$	0.25	-
Sequestrene Fe 330	40	40
	VITAMINS	
Myo-Inistol	100	100
Nicotininc acid	5	1
Pyridoxine HCl	0.5	-
Thiamine	0.5	2
Glycine	2	2
Folic acid	0.5	-
Biotin	0.05	-

Appendix 3.

Nutrient composition of the culture media:

Media	*Modified MS type A (mg/l)*	*Modified MS type B (mg/l)*
	MACROELEMENTS	
NH_4NO_3	1650	1650
KNO_3	1900	1900
$CaCl_2.2H_20$	440	440
$Ca(NO_3)_2.4H_2O$	-	-
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
K_2SO_4	-	-
KCl	-	-
	MICROELEMENTS	
KI	0.83	0.83
H_3BO_3	6.2	62
$MnSO_4.4H_2O$	22.3	223
$ZnSO_4.7H_2O$	8.6	86
$CuSO_4.5H_2O$	0.025	0.25
$Na_2MoO_4.2H_2O$	0.25	2.5
$CoCl_2.6H_2O$	0.025	0.25
$((NH_4)_2HPO_4)$		-
$NH_4H_2PO_4$		-
Sequestrene Fe 330	40	40
	VITAMINS	
Myo-Inistol	100	100
Nicotininc acid	1	1
Pyridoxine HCl	1	1
Thiamine	10	10
Glycine	-	-
Folic acid	-	-
Biotin	-	-

Appendix 4.

Nutrient composition of the culture media:

Media	*Modified OM type A (mg/l)*	*Modified OM type B (mg/l)*
	MACROELEMENTS	
NH_4NO_3	412	412
KNO_3	1100	1100
$CaCl_2.2H_20$	440	332.2
$Ca(NO_3)_2.4H_2O$	600	416.9
$MgSO_4.7H_2O$	1500	732.6
KH_2PO_4	340	340
K_2SO_4	-	-
KCl	500	500
	MICROELEMENTS	
KI	0.83	0.83
H_3BO_3	12.4	124
$MnSO_4.4H_2O$	22.3	169
$ZnSO_4.7H_2O$	14.3	143
$CuSO_4.5H_2O$	0.25	25
$Na_2MoO_4.2H_2O$	0.25	25
$CoCl_2.6H_2O$	0.025	0.25
Sequestrene Fe 330	40	40
	VITAMINS	
Myo-Inistol	100	100
Nicotininc acid	5	5
Pyridoxine HCl	0.5	0.5
Thiamine	0.5	0.5
Glycine	2	2
Folic acid	0.5	0.5
Biotin	0.05	0.05

Appendix 5.

The concentration of polyamines (nmol g^{-1} FW) measured in leaf sample from experiment 1 (section 4. 6. E.):

Table 1. The effect of culture regime on the mean putresine content (nmol g^{-1} FW) in leaf samples of genotypes S11, F5, 72 and 49 over 245 days.

Culture regime	1		2				3			
Genotype	*S11*	*F5*	*S11*	*F5*	*M72*	*JK49*	*S11*	*F5*	*M72*	*JK49*
Days in culture										
0	11.1*	7.1	11.1	7.1	66.6	46.3	11.1	7.1	66.6	46.3
17	6.5	10.9	9.7	20.5	79.4	6.8	9.6	20.5	79.4	6.8
35	17.7	9.2	26.0	132.8	238.6	25.6	26.0	132.8	238.6	25.6
52	9.4	11.2	221.5	244.0	815.5	338.9	464.3	145.1	136.2	488.7
70		11.8	79.5	229.4	161.7	193.7	533.4	8.3	294.92	160.6
87		5.4	1193.3	872.5	194.2	1064.9	510.1	667.5	733.32	2043.2
105		4.6	1052.8	65.7	114.6	290.8	1002.1	109.5	3429.8	264.1
140		4.9	-	1388.5	2756.1	1239.9	485.9	684.8	1355.8	1186.4
175		5.6	1134.7	1661.5	3041.0	1994.5	1179.3	938.2	468.5	-
210							639.8	513.0	740.1	759.1
245								1097.3	1630.0	260.4

* Each data point is the mean of three samples.

Table 2. The effect of culture regime on the mean spermidine content (nmol g^{-1} FW) in genotypes S11, F5, 72 and 49 over 245 days.

Culture regime	1		2				3			
Genotype	*S11*	*F5*	*S11*	*F5*	*M72*	*JK49*	*S11*	*F5*	*M72*	*JK49*
Days in culture										
0	7.0*	17.0	7.0	17.0	15.9	7.9	7.0	17.0	15.9	7.9
17	9.8	13.8	10.9	64.5	7.6	7.4	10.9	64.5	7.6	7.4
35	20.3	17.3	46.0	146.4	224.5	34.9	46.0	146.8	224.5	34.9
52	16.4	12.7	20.3	480.7	130.6	172.9	73.9	137.9	64.1	70.2
70		6.2	55.8	622.4	100.2	505.5	33.7	44.0	59.4	401.1
87		11.1	161.3	606.9	414.9	174.2	44.7	72.2	58.7	415.3
105		18.9	-	648.9	131.9	429.6	146.4	297.9	152.4	64.5
140		14.2	192.3	500.8	358.1	153.1	13.8	48.7	230.9	-
175		5.8	206.3	215.8	501.3	453.42	138.8	199.9	132.0	196.0
210							42.6	6.8	48.0	19.9
245								222.5	181.4	30.3

* Each data point is the mean of three samples.

Table 3. The effect of culture regime on the mean spermine content (nmol g^{-1} FW) in genotypes S11, F5, 72 and 49 over 245 days.

Culture regime	1		2				3			
Genotype	*S11*	*F5*	*S11*	*F5*	*M72*	*JK49*	*S11*	*F5*	*M72*	*JK49*
Days in culture										
0	4.7*	2.5	4.7	2.5	7.3	10.7	4.7	2.5	7.3	10.7
17	5.2	3.2	6.0	8.8	4.9	3.4	6.0	8.8	4.9	3.4
35	14.4	6.9	12.8	84.4	84.5	28.9	12.8	84.4	84.5	28.9
52	8.6	6.2	3.4	88.1	60.4	87.9	5.4	179.9	19.8	28.9
70		2.9	5.0	130.6	57.3	139.2	3.4	11.1	21.2	51.1
87		2.2	62.9	148.8	83.3	103.9	8.3	25.0	34.9	151.4
105		1.9	-	383.4	106.8	199.1	16.7	175.0	82.5	29.7
140		4.8	91.5	427.9	244.3	124.5	4.4	22.7	191.4	-
175		2.1	94.4	120.2	143.5	160.9	44.8	120.8	65.2	79.4
210							10.3	1.2	30.8	9.1
245							-	125.6	43.2	13.8

* Each data point is the mean of three samples.

Appendix 6.

Table 4. The allele lengths (base pairs) of vegetative propagules of genotype F5 for five microsatellite loci (a number in bold type signifies a different allele).

	Locus									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
Sample										
*M05A	171		187		184	200	**176**	**184**	202	217
M05B	**173**	208	187		184	200	190	210	202	217
M05C	171	208	187		184	200	190	210	202	217
M05D	171	208	187		184	200	190	210	**194**	**223**
C05A	171	208	187		184	200	190	210	202	**214**
C05B	171	208	187		184	200	190	210	**180**	**186**
C05C	171	208	187		184	200	190	210	**180**	**186**

* M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant.

Table 5. The allele lengths (base pairs) of vegetative propagules of genotype 8 for five microsatellite loci (a number in bold type signifies a different allele).

	Locus									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
Sample										
*K08A	172	197	187	197	185		172	200	208	229
K08B	172	197	-	-	185		**174**	**204**	208	229
K08C	172	197	187	197	185		172	200	208	229
K08D	172	197	187	197	185		172	200	208	229
F08A	172	197	187	197	185		172	200	208	229
F08B	172	197	187	197	185		172	200	208	229
F08C	172	197	187	197	185		172	200	208	229
G08A	172	197	187	197	185	**194**	172	200	208	229
G08B	172	197	187	197	185	**194**	172	200	208	229

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); G=Grafted (Kinsealy glasshouse).

Table 6. The allele lengths (base pairs) of vegetative propagules of genotype 18 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K18A	171		195	207	185		180	201	216	
K18B	171		195	207	185		180	201	216	
K18C	171		195	207	185		180	201	216	
K18D	171		195	207	185		180	201	216	

* K= Grafted (Kilmacurra).

Table 7. The allele lengths (base pairs) of vegetative propagules of genotype 22 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K22A	183		189		184	201	194	203	216	230
K22B	183		189	195	**180**		194	203	216	230
K22C	183		189	195	184	201	194	203	216	230
K22D	183		189	195	184	201	194	203	216	230
G22A	183		189	195	184	201	194	203	216	230
G22B	183		189	195		201	194	203	216	230
G22C	183		189	195		201	194	203	216	230

*K= Grafted (Kilmacurra); G=Grafted (Kinsealy glasshouse).

Table 8. The allele lengths (base pairs) of vegetative propagules of genotype 29 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K29A	169		189		184	**208**	191	196	212	225
K29B	169		189		-	-	191	196	212	225
K29C	169	**201**	189		184		191	196	212	225
G29A	169	**201**	189		184		191	196	212	225
G29B	169		189		184		191	196	212	225
G29C	169		189		184		191	196	**202**	**215**

* K= Grafted (Kilmacurra); G=Grafted (Kinsealy glasshouse).

Table 9. The allele lengths (base pairs) of vegetative propagules of genotype 44 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K44A	176	183	189	**207**	185	**201**	**180**	**194**	**199**	
K44B	176	183	189	228	185		190		220	
K44C	176	183	189	228	185		190		220	

*K= Grafted (Kilmacurra)

Table 10. The allele lengths (base pairs) of vegetative propagules of genotype 47 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K47A	168	**186**	189	197	184	200	195	200	223	241
K47B	168	191	189	197		200	195	200	223	241
K47C	168	191	189	197		200	195	200	223	241
K47D	168	191	189	197	184	200	195	200	223	241
F47A	168	**172**	189	197	184	200	195	200	223	241
F47B		191	189	197	184	200	195	200	223	241
F47C	168	191	189	197	184	200	195	200	223	241
M47A	**172**	**182**	**195**	**206**	**184**		**187**	**192**	**196**	**235**
M47B	**172**	**182**	**195**	**206**	**184**		**187**	**192**	**196**	**235**
M47C	**172**	**182**	**195**	**206**	**184**		**187**	**192**	**196**	**235**
C47A	**172**	**182**	**195**	**206**	**184**		**187**	**192**	**196**	**235**
C47B	**172**	**182**	**195**	**206**	**184**		**187**	**192**	**196**	**235**
C47C	**172**	**182**	**195**	**206**	**184**		**187**	**192**	**196**	**235**

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant.

Table 11. The allele lengths (base pairs) of vegetative propagules of genotype 49 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K49A	177	**200**	191		184	200			199	208
K49B	177	**200**	191		184		200		199	208
K49C	168	177	191		184	200	200		199	208
K49D	168	177	191		184	200	200		199	208
F49A	168	177	191		184	200	**194**	200	**215**	**245**
F49B	168	177	191		184	200	**202**		**215**	
F49C	168	177	191		184	200	**194**	200	**197**	**207**
M49A	**172**	**182**	**188**	**207**	184		**187**	**192**	**194**	**235**
M49C	**172**	**182**	**195**	**207**	184		**187**	**192**	**185**	
C49A	168	177	**188**		184		**202**		**208**	
C49B	**172**	**182**	**195**	**207**	184		**187**		**197**	
C49C	**172**	**182**	**195**	**207**	184		**187**	**192**	**197**	**235**

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant.

Table 12. The allele lengths (base pairs) of vegetative propagules of genotype 56 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K56A	175	**230**	195	224	184	200	**173**	**195**	214	245
K56B	**165**		195	224	184	200	193		214	245
K56C	**165**	**199**	195	224	184		193		**224**	**173**
K56D	175	**185**	195	224	184	200	193		214	245
F56A	175		195	224	184	200	193		214	245
F56B	175		195	224	184	200	**191**		**199**	**209**
G56A	175	**189**	195	224	184	200	**167**	**176**	214	245
G56B	**166**	**172**	195	224	184	200	**176**		214	245
G56C	175	**189**	195	224	184	200	**176**		214	245

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); G=Grafted (Kinsealy glasshouse).

Table 13. The allele lengths (base pairs) of vegetative propagules of genotype 69 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K69A	175	**184**	**187**	196	184	**191**	191		195	
K69B	**163**	175	**187**	196	184		**148**		**243**	
K69C	175	**193**	**209**		184		**182**		195	**209**
F69A	175		196		184		191		195	
F69B	175		196		184		191		195	
F69C	**184**		196		184		191		195	

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field).

Table 14. The allele lengths (base pairs) of vegetative propagules of genotype 70 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K70A	160		188	206	184		192		**232**	
K70B	**195**	**183**	**195**		184		**198**	**200**	**229**	
K70C	**171**		**191**	**195**			**190**		**204**	**217**
K70D	**165**	**178**	**191**	**195**	184	**200**	**190**		**217**	**230**
F70A	160	163	188	206	184		192		**195**	
F70B	160	163	188	206	184		192		**236**	
F70C	160	163	188	206	184		192		**236**	
M70A	**167**		188		184		**172**	**200**	**185**	
M70B	**167**		188		184		**172**	**200**	**209**	
M70C	**165**		188	**213**	184		**172**	**200**	**209**	
M70D	**165**		188	**213**	184		**172**	**200**	**209**	
C70A	**166**	**178**	188	**213**	184		**187**	192	**209**	
C70B	**166**	**178**	188	**213**	184		**200**		**209**	
C70C	**166**	**178**	188	**213**	184		**200**		**209**	

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant.

Table 15. The allele lengths (base pairs) of vegetative propagules of genotype 72 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K72A	166	**193**	187	195	184		**143**	**204**	**213**	
K72B	176		187	195	**196**		**208**		**196**	**247**
K72C	166	176	187	195	184		**190**	**208**	**194**	**208**
K72D	166	176	**188**	**213**	184		**172**	**202**	**210**	**213**
F72A	**171**	**183**	**194**	**207**	**185**		**188**	**191**	**195**	**234**
G72A	166	**256**	**186**	**196**	**185**	**212**	**167**	**180**	**210**	
G72B	166	**256**	**186**	**196**	184		**167**	**183**	**195**	**247**
G72C	166	**178**	**184**	**221**	184		**167**	**180**	**210**	
M72A	166		**188**	**213**	**161**	**185**	**172**	**200**	**209**	
M72B	166		**188**	**213**	184		**172**	**200**	**209**	
M72C	166		**188**	**213**	184		**172**	**200**	**209**	
M72D	166	**178**	**188**		184		**200**		**209**	
C72A	166	**178**	**188**	**213**	184		**200**		**209**	
C72B	166	**178**	**188**		184		**200**		**209**	
C72C	166	**178**	**188**	**213**	184		**200**		**209**	

* K= Grafted (Kilmacurra); F= Grafted (Kinsealy field); G=Grafted (Kinsealy glasshouse); M=micropropagated (weaned) plant; C= micropropagated (*in vitro*) plant.

Table 16. The allele lengths (base pairs) of vegetative propagules of genotype 73 for five microsatellite loci (a number in bold type signifies a different allele).

Sample	*Locus*									
	FEMSATL 4		FEMSATL 11		FEMSATL 16		FEMSATL 19		M2-30	
*K73A	166		**195**		185		**194**	**202**	**199**	**210**
K73B	166	173	189		185		187	191	223	229
K73C	166	173	189		185		187	191	**231**	
K73D	166	173	189		185		187	191	223	229
G73A	166		**187**	**191**	185		187	191	223	229
G73B	166		**187**	**191**	185		**176**		223	229
G73C	166		**187**	**191**	185		**167**		223	229

* K= Grafted (Kilmacurra); G=Grafted (Kinsealy glasshouse).