

MICROPROPAGATION OF CALABRIAN PINE

(*PINUS BRUTIA* TEN.)

ANWAR A. ABDULLAH

DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH

1987



Table Of Contents

Title page.....	I
Declaration.....	II
Table of contents.....	III
Acknowledgement.....	IX
Publications.....	X
Abstract.....	XI
Abbreviations and symbols.....	XII
Terminology.....	XIV
Chapter 1 Introduction.....	1
1.1 Importance of the species.....	2
1.2 Traditional methods for tree propagation and improvement.....	3
1.3 Micropropagation of gymnosperms.....	7
1.3.1 <i>Choice of nutrient medium</i>	10
1.3.2 <i>The physical culture environment</i>	14
(i) Physical properties of the nutrient medium.....	15
(ii) Light.....	17
(iii) Temperature.....	18
1.3.3 <i>Influence of growth substances</i>	20
1.3.4 <i>Selection of explants</i>	30
1.3.5 <i>Regulation of the development process</i>	37
1.3.6 <i>In vivo performance of tissue culture plantlets</i>	44
1.3.7 <i>Some economic considerations</i>	47
1.4 Statement of objectives.....	47
Chapter 2 Materials and methods.....	48
2.1 Plant Material.....	49
2.1.1 <i>Seedling-plant material</i>	49
(a) Sources.....	49
(b) Excision of explants.....	49
2.1.2 <i>Mature plant material</i>	50
(a) Sources.....	50
(b) Collection of explants.....	50
(c) Excision of explants.....	50

2.2	Nutrient Medium.....	51
2.2.1	<i>Choice and modification to the nutrient medium....</i>	51
2.2.2	<i>Preparation of stock solution for the nutrient medium.....</i>	51
2.2.3	<i>Preparation of stock solution for growth substances.....</i>	53
2.2.4	<i>Preparation of other additives.....</i>	53
2.2.5	<i>Preparation of the nutrient medium and cytokinin solution.....</i>	54
2.3	Sterilization Techniques.....	56
2.3.1	<i>Sterilization by heat.....</i>	56
2.3.2	<i>Sterilization with hypochlorite-peroxide.....</i>	56
2.3.3	<i>Sterilization with ethanol-mercuric chloride.....</i>	57
2.3.4	<i>Sterilization with ethanol.....</i>	57
2.3.5	<i>Sterilization by ultra-violet light.....</i>	57
2.4	Culture Conditions.....	61
2.4.1	<i>Bud initiation.....</i>	61
	(a) Culture medium and containers.....	61
	(b) Environmental conditions.....	61
2.4.2	<i>Bud elongation and shoot formation.....</i>	62
	(a) Nutrient medium and containers.....	62
	(b) Environmental conditions.....	62
2.4.3	<i>Rooting treatment and root induction.....</i>	62
	(a) Nutrient medium and containers.....	62
	(b) Environmental conditions.....	63
2.4.4	<i>Hardening off and establishment of plantlets.....</i>	63
	(a) Nutrient medium and containers.....	63
	(b) Environmental conditions.....	64
2.5	Culture Routine.....	65
2.5.1	<i>Adventitious shoot formation on cultured of seedling-needles.....</i>	65
	(a) Initiation of adventitious buds.....	65
	a.1 Cytokinin treatments.....	65
	a.2 Age of donor seedlings.....	66
	a.3 Physical state of applied medium.....	66
	a.4 Final account using optimal procedure.....	66

(b) Bud development and shoot formation.....	67
(c) Bud multiplication.....	67
2.5.2 Axillary shoot formation on seedling-shoot explants.....	68
(a) Axillary bud and shoot development.....	68
a.1 Application of cytokinins.....	68
a.2 Surgical removal of apical buds.....	68
(b) Shoot formation from secondary buds.....	68
(c) Shoot multiplication.....	69
2.5.3 Formation of shoot-buds on mature fascicle needles.....	69
(a) Initiation of shoot-buds.....	69
a.1 Prevention of browning of explants.....	69
(i) Soaking of explants before culture.....	69
(ii) Application of anti-browning substances to culture media.....	70
(iii) Application of anti-browning substances to culture of seedling-needles.....	70
(iv) Application of different levels of sodium diethyldithiocarbamate to cultures of seedling-needles.....	71
(v) Application of sequence-treatments to cultures of mature fascicle needles.....	72
a.2. Effects of season of collection.....	73
(b) Further shoot elongation and multiplication.....	73
2.5.4 Regeneration of plantlets.....	73
(I) Adventitious root formation on axillary-derived shoots.....	73
(a) Application of growth substances.....	74
a.1 Application of various types and concentrations of auxins.....	74
a.2 Application of different concentrations of cytokinin.....	74
(b) Application of different root-induction media.....	75
(II) Adventitious root formation on adventitious-derived shoots grown from seedling-needles.....	76

(III) Adventitious root formation on shoots induced on mature fascicle needles.....	76
2.5.5 <i>Patterns of growth and development of plantlets</i> ...	77
1. Axillary plantlets.....	77
2. Adventive plantlets.....	77
3. Seedlings.....	77
2.6 Determination of some growth characteristics of plants.....	79
2.6.1 <i>Dry weight of the plants</i>	79
2.6.2 <i>Specific leaf area and leaf dry weight ratio</i>	79
2.6.3 <i>Stomatal resistance of leaves</i>	79
2.6.4 <i>Patterns of growth form of plantlets</i>	80
2.7 Anatomical Investigations and Scanning electron microscopy (SME).....	81
2.8 Statistical analysis.....	82
Chapter 3 Experimental Results.....	83
3.1. The production of buds and shoots.....	84
3.1.1 <i>Adventitious shoot formation on cultured seedling-needles</i>	87
A. Initiation of adventitious buds.....	87
i. Anatomical observations with the light and scanning electron microscope.....	87
ii. Influence of growth substances.....	90
a. Influence of cytokinins.....	90
b. Influence of mode of application of cytokinin.....	92
c. Influence of inclusion of auxin with applied cytokinin.....	92
iii. Influence of physical properties of nutrient medium.....	92
vi. Influence of age of donor seedlings.....	96
B. Multiplication of induced adventitious buds.....	96
i. Influence of cytokinin treatments.....	96
C. Shoot formation from induced adventitious buds..	100
i. Influence of activated charcoal.....	100
D. Final account.....	100

3.1.2	<i>Axillary shoot formation on seedling-shoot explants</i>	101
	A. Induction and development of axillary buds and shoots.....	101
	i. Anatomical observations with the light and scanning electron microscope.....	101
	ii. Shoot formation from primary axillary buds...	101
	iii. formation of secondary buds.....	103
	B. Shoot-bud multiplication.....	103
	C. Shoot elongation.....	107
	D. Axillary shoot formation on explants of shoots from 6-month-old seedlings.....	107
3.1.3	<i>Formation of shoot-buds on mature fascicle needles</i>	108
3.1.3.1	<i>Initiation of shoot-buds</i>	108
	A. Influence of anti-browning substances.....	108
	i. Rate of oxidative browning on cultured fascicles.....	108
	ii. Morphogenic responses of excised needles from seedlings.....	111
	B. Influence of season of collection.....	111
	i. Rate of oxidative browning of cultured fascicles.....	111
	ii. Morphogenic responses of cultured fascicles...	111
	C. Influence of sequential treatments.....	111
3.1.3.2	<i>Elongation of induced shoot-buds</i>	115
3.1.3.3	<i>Multiplication of elongated shoot-buds</i>	115
3.2	<i>The regeneration and establishment of plantlets</i>	118
3.2.1	<i>Regeneration of plantlets</i>	121
	I. Adventitious root formation on axillary-derived shoot	
	A. Influence of various types and concentrations of growth substances.....	121
	i. Induction of adventitious roots.....	121
	ii. Rate of root elongation and the number of roots produced.....	121
	iii. Branching of induced roots.....	124

iv. Rate of growth shoot height [↓] (mm/month).....	129
v. Survival of established plantlets.....	129
B. Influence of different root induction media.....	129
i. Quantitative and qualitative characteristics of induced roots.....	129
ii. Callus growth.....	133
iii. Survival of rooted shoots.....	133
II. Adventitious root formation on adventive shoots grown from seedling-needles.....	133
III. Adventitious root formation on shoots induced on mature fascicle needles.....	133
3.2.2 <i>Establishment of plantlets</i>	134
A. Growth characteristics of the plants.....	134
B. Growth characteristics of fascicles.....	138
C. Growth patterns and form of plants.....	138
Chapter 4 Discussion and conclusions	143
4.1 Nutrient medium.....	144
4.2 Physical culture environment.....	148
4.3 Influence of growth substances.....	155
4.4 Influence of explant source.....	164
4.5 Regulation of the development process.....	171
4.6 General conclusion and prospects.....	176
Bibliography	183

Acknowledgements

I would like to thank the numerous people who were involved in this work in one way or another. I sincerely thank my supervisors *Dr. John Grace* of FNR Department and *Professor Michael M. Yeoman* of the Botany Department, for their support & guidance, stimulating discussions and fruitful advice throughout the project. I wish to record my sincere appreciation to *Jeremy Landless* for construction and maintenance of the growth chamber and for his technical help at all times. I would like to express my gratitude to *Dr. Chris Jeffree* for his helpful advice during microscopy & EM studies. I thank *Dr. Chris Page* & *Mr. G. Broadley* for their interest and help in providing plant material from The RBG, Edinburgh. I would like to express my thanks to *David Haswell* for the photography and line drawings. Thanks are also due to *Alex Harrower* & *David Mackenzie* for their assistance in the workshop. I am also grateful to *Bob Astles* for looking after my plants in the greenhouse. I am indebted to *Alison Wood*, librarian of FNR and to *Miss. H. Quinn* in the Botany Department, for their help in obtaining many of the more obscure references. Special thanks for *Professor Paul Jarvis* for furnishing many of the references. I also acknowledge, with thanks, the skilled technical assistance of *John Findlay* with preparation of specimens for microscopy & EM studies. I wish to express thanks to *Margaret Jackson* for typing the manuscript. The encouragement of the staff and colleagues in FNR & Botany Department is acknowledged with sincere thanks. Finally, I would like to dedicate this thesis to my wife & daughters who offered full support and encouragement throughout the study. The financial support for this project from the Ministry of High Education, Government of Iraq, is also gratefully acknowledged.

Publications

- Abdullah, A.A., Yeoman, M.M. & Grace J. (1984). *In vitro* organogenesis of *Pinus brutia* needles and shoot tips. In Proc Intern. Symp. on Plant Tissue and Cell Culture Application to Crop Improvement, September 24-29 1984, Czechoslovakia. F.J. Novak, L. Havel and J. Dolezel (eds), pp. 177-178. Czechoslovak Academy of Sciences, Prague, Czechoslovakia.
- Abdullah, A.A., Yeoman, M.M. & Grace, J. (1985). *In vitro* adventitious shoot formation from embryonic and cotyledonary tissues of *Pinus brutia* Ten.. *Plant Cell Tissue Organ Cult.* 5: 35-44.
- Abdullah, A.A., Grace, J. & Yeoman, M.M. (1986). Rapid micropropagation of Calabrian pine from primary and secondary buds on shoot explants. *Can. J. For. Res.* 16: 637-641.
- Abdullah, A.A., Yeoman, M.M. & Grace, J. (1987). Calabrian pine (*Pinus brutia* Ten.) In *Biotechnology in Agriculture and Forestry, Trees II*. Y.P.S. Bajaj (ed.), Springer-Verlag, Berlin-New York. (In Press).
- Abdullah, A.A., Yeoman, M.M. & Grace, J. (1987). Micropropagation of mature Calabrian pine (*Pinus brutia* Ten.) from fascicular buds *Tree Physiol.* (In Press).

Abstract

Methods were developed for micropropagation of Calabrian pine (*Pinus brutia* Ten.) using seedling and mature-tree explants. Adventitious buds were induced on seedling-needles using a medium containing a cytokinin. An increase in the production of buds appears to depend on the type and concentration of cytokinin employed, whether auxin and cytokinin are added together, and the physical state of the nutrient medium. The mode of application of cytokinin and the influence of age of the donor seedling were also important. A method was described for the production of axillary buds using excised shoot pieces from seedlings, in which the production was increased by regulating the cytokinin level and by the removal of apical buds from the cultured explants. An analysis was made of the gross anatomical changes and the developmental sequence of adventitious and axillary bud formation on cultured explants. A procedure for the activation of shoot-buds on fascicles taken from mature trees was also established. This involved the use of a method to prevent browning of the cultured fascicles and their collection at a favourable time of year. In all cases, development of the induced buds into distinct shoots occurred when the explants bearing induced organs were transferred onto a medium, either lacking cytokinin, or with reduced level, and also on a medium with activated charcoal. Multiplication of the buds was obtained by maintaining these buds on a medium which included cytokinins. Rooting of the elongated shoots was achieved on an appropriate combination of two auxins and a cytokinin. Survival and growth of plantlets in soil was influenced by the concentrations of growth substances that had been initially used for root induction, the origin of the induced shoots and the media employed for root induction. The results are discussed in relation to a strategy for a tree improvement programme.

Abbreviations and symbols

- AC : Activated charcoal.
 ASA : L- Ascorbic acid 'AnalaR'.
 BAP : 6-benzylaminopurine.
 CK : Cytokinin.
 CV : Coefficient of variation.
 cm : Centimeter.
 d : Day.
 DNA : Deoxyribonucleic acid.
 g : Gram.
 GD : Gresshoff and Doy (1972)
 h : Hour.
 H_2O_2 : Hydrogen peroxide.
 IAA : Indole-3-acetic acid.
 IBA : Indole-3-butyric acid.
 ZiP : 2-isopentenylpurine.
 Insol. PVP: Insoluble Polyvinylpolypyrrolidone.
 Kin : Kinetin.
 L_A : Leaf area (cm^2).
 LM : Light microscopy.
 LSR : Least significant range.
 LS : Linsmaier and Skoog (1965).
 L_w : Leaf dry weight (g).
 M : Molar.
 m : Meter.
 MCA : Malonic acid (Propanedioic acid).
 min : Minute.
 ml : Millilitre.
 mm : Millimeter.
 mon : Month.
 MS : Murashige and Skoog (1962).
 μm : Micrometre.
 μmol : Micromole.
 NAA : 1-Naphthalenacetic acid.
 nm : Nanometre.
 pH : Negative logarithm of the hydrogen concentration.
 PPZ : 3-Methyl-1-phenyl-5-pyrazolone.

psi : lb/ inch².
rps : Rotation per second.
s : Second.
SC : Stored clones.
SDD : Sodium diethyldithiocarbamate.
SEM : Scanning electron microscopy.
SH : Schenk and Hildebrandt (1972).
SLA : Specific leaf area and dry weight ratio.
sol. PVP: Soluble polyvinylpyrrolidone.
TC : Tested clones.
v/v : Volume per volume.
W : Watt.
w/v : Weight per volume.
yr : Year.
ZEA : Zeatin.

Terminology

There follows an alphabetical index of terms which are commonly used in the literature relating to the tissue culture of conifers, and in tissue culture more generally. The definitions are those which apply to the work reported in this thesis.

Additive gene effects: Are due to the cumulative effects of alleles at all gene loci, influencing a trait, such as wood specific gravity, bole straightness and other quality characteristics of trees. Its presence contributes to the 'general combining abilities' of the selected parents, where performance of the offspring is usually predicted by and similar to the performance of the parents.

Adventitious: Tissues or organs developing in an abnormal position, i.e. buds produced at places other than from leaf axil and roots growing from stem or leaves.

Auxins: Plant growth regulators characterized as substances which are involved in, cell enlargement, apical dominance and root initiation. One natural auxin is IAA (Indoleacetic acid).

Axillary bud: A bud found in the axil of a leaf.

Brachyblast: Used to describe the bundle of 2-10 needles and associated structures that occur in the genus *Pinus*, also called fascicles, short shoot, or branch bearing leaf tuft; dwarf spur; specifically refers to the suppressed bud-like structure of meristematic, quiescent tissue; extended by secondary, linear leaves borne in fascicles and surrounded at the base by sheath of bud scales.

Bud: An undeveloped shoot covered with protecting scales, consists of a very short shoot axis and primordia of leaves or floral parts.

Callus: Actively growing relatively undifferentiated tissue, devoid of macroscopic organized structures, normally produced in higher plants in response to wounding or infection, but often formed *in vitro* during the artificial culture of plant tissue.

Chelating agent: An organic compound that is capable of complexing with a metal ion to form a chemical structure in which a central polyvalent metal ion is combined with a ring of organic compounds or radicals.

Clonal propagation: Asexual propagation from the same original cells

or organism (ortet) of many new plants (ramets) all with the same genome or genetic material.

Cytokinin: Plant growth regulators characterized as substances which are involved in cell division and dedifferentiation. Zeatin is a natural cytokinin.

Dedifferentiation: The losing of characteristics of specialized cells and regression to a more simple state. Often has the meaning of reprogramming of cells to a state in which they are capable of adventitious organ or embryo formation.

Development: Qualitative change undergone by organism via differentiation and growth from its beginning to maturation.

Differentiation: The general process by which unspecialized cells change in structure and function to perform a particular function in the tissues or organs of an organism.

Embryogenesis: Process of embryo initiation and development to an autotrophic plant from a cell culture.

Explant: Original part of the parent plant tissue that is introduced to *in vitro* conditions.

Genotype: Genetic constitution of an individual carried in the DNA of the cell.

Growth: An irreversible increase in volume or mass associated with the development. It usually involves cell division, expansion, differentiation and morphogenesis.

Heterotrophic: Of an organism which requires a supply of a carbon compound as a source of energy and for growth. Such organisms usually cannot fix carbon dioxide in the light.

Heterozygous: An organism or cell having 2 different alleles at corresponding loci on homologous chromosomes.

In vitro: A sterile, artificial environment, typically in glass vessels, in which cultured cells, tissues, organs or whole plants may reside.

In vivo: The natural conditions in which organisms reside.

Meristemoid: An active locus of growth; a nodule of undifferentiated tissue from which new cells and/or adventitious structures arise.

Micropropagation: Rapid vegetative propagation of a plant via small pieces of tissue, and usually beyond that obtained in nature.

Non-additive gene effects: Are due to specific combinations either the interactions of specific alleles at a gene locus or the interactions among gene loci. Performance of the offspring is neither predicted by nor similar to that of the parents.

Organogenesis: Initiation of an organ, or the production of a plantlet *in vitro* through the sequential usually non-synchronized initiation of root and shoot structures connected by vascular tissue.

Plantlet: A tiny plant with a distinct root and shoot system formed via tissue culture either by embryogenesis or organogenesis.

Somaclonal variation: Genetic, epigenetic and phenotypic variation among somatic cells and micropropagules, regarded as a result of genetic differences preexisting in somatic cells or induced by the environment within the culture medium.

Tissue Culture: A general term used to describe the development of tissues in culture under sterile conditions.

Totipotency: The ability of a somatic cell *in vitro* to regenerate a whole organism either via organogenesis or embryogenesis.

Vegetative propagation: Somatic nonsexual propagation of plant parts without fertilization.

Vitrification: A physiological disorder associated with specific changes in the appearance of induced organ *in vitro*, where leaves become translucent, appearing glassy or water logged and their needles adhered to each other.

Chapter 1

Introduction

1.1 IMPORTANCE OF THE SPECIES

Calabrian pine (*Pinus brutia* Tenore) is native to the Mediterranean Basin, where, along with the Aleppo pine (*Pinus halepensis* Mill.) it constitutes large forests. However, *Pinus brutia* has a more restricted range and a more easterly distribution than *Pinus halepensis* (Dallimore and Jackson 1954, Dives 1965, Mirov 1967). Calabrian pine grows in the Italian province of Calabria (ancient Brutium), where it was apparently planted a long time ago, perhaps even before the Romans (Mirov 1967, Mirov and Hasbrouck 1976). In its natural habitat in the eastern Mediterranean region, *Pinus brutia* occupies some 40000 km² (Le Houerou 1981), including the south Aegean (on the islands of Chiose, Samos, Rhodes, and Crete), the Crimea, N. Iraq, W. Syria, the Lebanon, S. Turkey (Taurus mountains), Cyprus, and Greece (Davis 1965, Anders and Kettaneh 1969, Kayacik and Yaltirik 1970).

Pinus brutia is of increasing importance throughout the region for timber used in the construction industry, fuel production, and as a source of turpentine and resin. It can grow to 20-27 m, and is often tall and straight, and thus of great commercial value. The trees can be found in an altitudinal range of 100-1500 m, occasionally reaching 1550 m (Mirov 1955; 1967). Forests of this species in the eastern Mediterranean are relatively tolerant of climate, and are found in the humid, sub-humid, and semi-arid zones (Nahal 1981). This species seems to be indifferent to the soil quality, thriving on shallow limestone soil, tertiary gravels, sandstone formations, and calcareous marls (Dallimore and Jackson 1954, Anders and Kettaneh 1969, Boomsma 1979, Quezel 1981). The ability of *Pinus brutia* to grow in diverse climates and on various soil types is an advantage, which together with its good

form, is the reason it is preferred and used extensively in afforestation and reafforestation of the region (Anders and Kettaneh 1969, Kayacik and Yaltirik 1970). Beside its commercial use *Pinus brutia* is valued, in the areas where it has been introduced (as in Israel, Iran, and Australia), as an ornamental, a source of shade, a windbreak, and for erosion control (Webb 1974, Boomsma 1979).

One of the outstanding problems in silviculture and domestication of *Pinus brutia* is the lack of adequate methods of vegetative propagation. *Pinus brutia* can only be propagated by seed, and as this species is an outbreeder, highly variable progeny are produced. Indeed, seed orchards have been established in the regions as a part of a tree improvement plan (Michaelides 1978), but so far, apart from the expense, this approach has been restricted by biological problems (Libby 1979). In Greece, hybrids of Calabrian pine and Aleppo pine have been obtained by artificial crossing (Moulopoulos and Bassiotis 1963). However, the long reproductive cycle places severe limitations on such traditional approaches. It seems now that these long established methods for tree propagation and improvement, which are costly, inefficient, and unable to produce trees of improved quality, are no longer acceptable. Thus, new methods for tree improvement of this species are required. Tissue and organ culture techniques provide a promising and alternative approach to the traditional methods.

1.2 TRADITIONAL METHODS FOR TREE PROPAGATION AND IMPROVEMENT

Perhaps the primary constraint on progress in most tree improvement programmes is the length of the breeding cycle. It is for this reason that many tree crops are virtually as variable as wild populations. The breeding strategy is to exploit this genetic variation using seed

orchards and controlled crossing, but the rate of improvement by this mode will always be much slower than in agricultural crops, where the life cycle is usually a few months instead of many years. Asexual or vegetative propagation (cloning) of trees is a useful tool in traditional tree improvement (Libby 1986). This method has the advantage of producing many copies of preferred genotypes. In the wild, there may be difficulties in ascribing genotypic, as opposed to environmental, causes of vigour and good form, because of environmental heterogeneity; but, this problem can be overcome by planting clones in trial areas as a first stage. Line breeding has an important role in advancing genetically the base population, and providing new and better material for cloning (Zobel and Talbert 1984). Subsequently, progress with vegetatively propagated species is influenced by the strategy used for deploying clones. It has been suggested that using a mixture of 20 clones may be ideal to give maximum genetic gains in vigour, while minimizing risks of disaster from insects or diseases (Libby 1979). Vegetative propagation of trees has been generally applied for several reasons: (1) The gain arising from any generation of a tree improvement programme can readily be multiplied for testing in several sites and for future commercial production. (2) To capture both additive and non-additive genetic effects within a population; the additive effect increases the selection frequency of favourable genes within individuals of a population, and non-additive effect ensure the cloning of superior individuals allowing the exploitation of the additional gain produced by the non additive gene effects in those individuals which may not have occurred in other siblings, and which, quantitatively, may be higher than the average value for all the

progeny in the family. (3) Genetically uniform parent plants are required for large-scale hybrid seed production. (4) Selected clones can be maintained in a 'gene bank' represented by a clonal orchard. Then genes can be recombined via controlled pollination. (5) By using pedigree clones, it is possible to prevent any inbreds, including selfs, from being planted in a production plantation. (6) Accurate information about genotype-environment interactions, as well as genetic and environmental covariance between characteristics can be obtained through carefully planned clonal studies. Also intra-clonal studies can provide more information about competition between planted adjacent trees, host-pathogen relations, and mycorrhizal symbiosis (Hussey 1978, Karnosky 1981, Libby 1979; 1986, Libby and Rauter 1984, McKeand and Weir 1984, Zobel and Talbert 1984).

Currently, rooting of cuttings is the most common practice of vegetative propagation in conifers, but in many species, this method has proved difficult. Perhaps the major dilemma with rooted cuttings of conifers is the rapid loss in rooting capacity with increasing age of the parent tree. Ironically, when the time is appropriate to select elite mature trees on the bases of their good past performance, propagation by cuttings is most difficult. Furthermore, speed of rooting, root length and number, survival and growth, all decline when the parent plant is more than 10 years of age (Girouard 1974). Many phenomena need a clearer scientific understanding if these problems are to be solved, e.g. the physiological causes responsible for differences in the rooting response of cuttings taken from various parts of the tree are not known. Also there are other awkward variables, i.e. the time of sampling of the explants, and the seasonal

variation in rooting capacity, which make this technique quite problematical. Furthermore, the propagules often retain their plagiotropic behaviour for a prolonged period, and may even display slower volume growth as the age of the ortet increases (Libby 1974, Bonga 1982_a). On the other hand, grafting is not sensitive to the effect of maturation of the donor trees, though it has been used less frequently for the vegetative propagation of conifers. This is particularly because grafting is labour intensive, and therefore, expensive (Libby 1979; 1986). Perhaps of equal importance is that graft incompatibility can be a serious problem, especially as rejection may not become evident for several years (Copes 1980, Yeoman et al. 1978, Yeoman 1984).

The outlook seems to be that the traditional methods of vegetative propagation in gymnosperms still suffer from many problems. Among these are the low efficiency and difficulties in propagating mature trees.

The world-wide demand for wood as an industrial raw material (for building, paper,.....etc.) is estimated to be increasing by 3 to 4% annually, and is likely to continue to do so at least until the year 2000 (Stockman 1980). Also demands for wood and its derivatives will increase for use in the plastic industry and fuel, either directly or indirectly (Karnosky 1981). Therefore, the predicted world-wide shortage of forest products portends an urgent need to increase forest productivity. Apparently, the traditional methods used for forest tree propagation and improvement (cutting, grafting) are not adequate to meet these demands. Therefore, new techniques to supplement the traditional methods must be developed. The emerging application of *in*

in vitro techniques provide an obvious choice as they offer the potential to clone and produce many plants rapidly on a very large scale (Murashige 1978, Sharpe and Evans 1981). Also they provide increased homogeneity and thus greater control over development in cells and tissues than with the traditional methods. Apparently the traditional methods are considerably affected by seasonal constraints, *in vitro* methods may circumvent this obstacle and thus offer a reliable means for annual propagation.

1.3 MICROPROPAGATION OF GYMNOSPERMS

Since gymnosperms constitute the major forest crop in many parts of the world, special attention has been given to *in vitro* culture of these species over the last seven decades. Almost, every review of the literature on plant tissue and organ culture begins with Haberlandt (1902) who was the first to put forward the concept of the totipotentiality of the plant cell, which is the ability to regenerate a whole organism from a single cell. This concept is central to the art of growing, aseptically and heterotrophically, isolated plant parts as explants on appropriate media to obtain organs and plantlets *de novo*. However, in gymnosperms, half a century was required to bring this methodology into practice. It was the work of Ball in 1950 who obtained buds from *Sequoia sempervirens*, which marked the start of an intensive interest in the *in vitro* culture of conifers. Another milestone occurred when the first gymnosperm plantlet was produced *in vitro* from embryonic tissues of *Pinus palustris* (Sommer et al. 1975). A resume of all the *in vitro* work carried out with gymnosperms over the period 1924-1974 was published by Brown and Sommer in 1975. Since then

Table 1.1. The regeneration of Conifers *in vitro* since 1975, with examples of the parts and ages of the species used as explants.

Species	Explants & age	References
<i>Pseudotsuga menziesii</i>	Embryos (--)	Winton & Verhagen (1977).
	Cotyledons (2-4 w)	Cheng (1975; 1977; 1979), Cheng & Voqui (1977).
	Needles (2-3 mo)	Reilly & Brown (1976), Chalupa (1977 _b).
	Stem segment + buds (2-60 yr)	Boulay (1976) Chalupa (1977 _a), Thompson & Zaerr (1982), Gupta & Durzan (1985).
<i>Tsuga heterophylla</i>	Cotyledons (2-5 w)	Cheng (1976).
<i>Sequoia sempervirens</i>	Stem segment + buds (20-100 yr)	Boulay (1979 _b).
<i>Thuja plicata</i>	Cotyledons (10-14 d)	Coleman & Thorpe (1977).
	Shoot tips (4-10 yr)	
<i>Biota orientalis</i>	Hypocotyls (2-4 w)	Thomas et al. (1977).
<i>Abies balsamea</i>	Buds (15-29 yr)	Bonga (1977; 1981).
<i>Picea abies</i>	Cotyledons (2-3 w)	Bornman & Jansson (1982).
	Embryos (--)	Von Arnold & Eriksson (1978).
	Needles (2-4 mo)	Von Arnold & Eriksson (1979 _b), Jansson & Bornman (1980; 1981), Bornman (1983).
	Buds (2-120 yr)	Chalupa (1977 _b), Von Arnold & Eriksson (1979 _a), Von Arnold (1984), Jansson & Bornman (1983)
<i>Picea glauca</i>	Hypocotyls (6-12 d)	Durzan & Campble (1976).
	Epicotyls (6-12 d)	Rumary & Thorpe (1984).
<i>Picea mariana</i>	Epicotyls (28 d)	Rumary & Thorpe (1984).
<i>Picea sitchensis</i>	Embryos (--)	Webb & Street (1977).
	Shoot apics (2 yr)	
	Buds (6-33 yr)	Selby & Harvey (1985).
<i>Larix decidua</i>	Cones (25-30 yr)	Bonga (1984).

Table 1.1 (Continued)

Species	Explants & age	References
<i>Pinus brutia</i>	Embryos (--) Cotyledons (4 d)	Abdullah et al (1985).
<i>Pinus contorta</i>	Embryos (--) Cotyledons (6-8 d)	Webb & Street (1977), Von Arnold & Eriksson (1981), Patel & Thorpe (1984). Patel & Thorpe (1984).
<i>Pinus lambertiana</i>	Stem seg- (8-30 yr) ment + buds	Gupta & Durzan (1985).
<i>Pinus monticola</i>	Embryos (--)	Mott & Amerson (1981 _b), Amerson & Mott (1982).
<i>Pinus nigra</i>	Embryos (--) Shoot apics (1-2 yr)	Jelaska et al. (1982).
<i>Pinus palustris</i>	Embryos (--)	Sommer et al. (1975).
<i>Pinus pinaster</i>	Cotyledons (8-10 d) Fascicles (2-3 yr) Brachyblasts (3-11 yr)	David et al. (1982). David et al. (1978). David (1979).
<i>Pinus radiata</i>	Embryos (--) Needles (7.5 mo) Cotyledons (5-7 d) Shoot apics (5-8 w)	Reilly & Washer (1977), Horgan & Aitken (1981). Reilly & Brown (1976), Aitken et al (1981;1982). Horgan & Aitken (1981).
<i>Pinus rigida</i>	Embryos (--) Cotyledons (7-10 d)	Patel et al. (1986).
<i>Pinus strobus</i>	Embryos (--)	Minocha (1980).
<i>Pinus sylvestris</i>	Cotyledons (2 w) Brachyblasts (5 mo) Embryos (--) Hypocotyls (5-7 d)	Tranvan (1979), Bornman & Jansson (1982). Bornman & Jansson (1980) Toribio & Pardos (1982).
<i>Pinus taeda</i>	Cotyledons (5-7 d) Fascicles (5 yr)	Mehra-Palta et al. (1978).
<i>Pinus williciana</i>	Embryos (--)	Konar & Singh (1980).

Abbreviation: d, day; mo, month; yr, year.

much progress has been made, and a large number of reports have appeared of the ~~the~~ regeneration *in vitro* of several economically important conifers. These are summarized in Table 1.1.

Most of the efforts put into the micropropagation of forest trees have been directed toward manipulating the regeneration process of cultured tissues through the appropriate choice of a nutrient medium (including the balance of applied growth substances), selection of suitable explants and the control of the physical culture environment. Since the theme of this study is the micropropagation of a conifer species, this chapter will concentrate exclusively on the literature relating to conifers. However, where the literature does not provide the required information, I will refer to studies carried out with other species.

1.3.1 Choice of nutrient medium

Micropropagation of gymnosperms has been generally performed on culture media identical to or modified from those originally formulated for the active growth of angiosperm callus tissues. Among the media which have found very wide application are those of Murashige and Skoog (MS) (Murashige and Skoog 1962), and Schenk and Hildebrandt (SH) (Schenk and Hildebrandt 1972). Both MS and SH media are characterized by having a high-salt level, but they are considerably different in composition. MS medium and its derivative by Linsmaier and Skoog (LS) (Linsmaier and Skoog 1965) contain a high level of nitrogen as ammonium. It had been reported that an ample supply of ammonia can reduce cell growth in some plant cultures (Gamborg and Shyluk 1970). Further support for this contention has been obtained from recent studies *in vitro*. Thompson and Zaerr (1982) found that the level of

NH_4NO_3 and in particular the high concentration of the ammonium ion in MS medium appears to be responsible for the inhibition of adventitious bud formation in Douglas fir cultures. This finding was confirmed with *Pinus williciana* (Konar and Singh 1980). However, Thompson and Zaerr (1982) also pointed out that satisfactory results were achieved when MS medium with 1/4 strength KNO_3 and NH_4NO_3 was applied. Also the recent advances in micropropagation of *Pseudotsuga menziesii* (Cheng 1979) and *Pinus nigra* (Jelaska et al. 1982) were achieved using a dilution of MS medium at 1/2 strength.

On the contrary, SH medium at full strength has been widely used for the culture of many conifer species e.g. *Pinus radiata* (Horgan and Aitken 1981), *Picea abies* (Jansson and Bornman 1983), *Picea glauca* and *Picea mariana* (Rumary and Thorpe 1984). Currently, the low iron concentration in SH medium is thought to be the reason for the production of yellow shoots in cultures of *Pinus radiata* (Horgan and Aitken 1981) and *Pinus brutia* (Abdullah et al. 1985). On the other hand, a low salt formulation, namely the medium devised by Gresshoff and Doy (GD) (1972) has also been applied successfully for the culture of many conifers e.g. *Pinus palustris* (Sommer et al. 1975), *Pinus taeda* (Mehra- Palta et al. 1978), *Pinus sylvestris* (Bornman and Jansson 1980), *Pinus monticola* (Mott and Amerson 1981_b). GD medium has approximately half the total salt concentration but double the iron level of SH medium.

Certainly, the success of *in vitro* techniques and their application will depend on a better understanding of the nutritional requirements of the cultures. A critical review of data available hitherto indicate that except for a few reports (e.g. Horgan and Aitken 1981, Bornman

1983, Abdullah et al. 1985) the media used have not as yet been critically evaluated for their intended purpose. Also there is a lack of information on the role of minerals ions in the control of plant development. So far, it appears that high potassium promotes organogenesis (David et al. 1978, David 1982). In this regard it is interesting to note that the highest concentrations of potassium are often found in the meristematic regions of the plant (Davlin 1975). Also it has been suggested that the concentration of calcium and its ratio to other macroelements in the culture media is of importance for shoot formation in *Picea abies* (Bornman 1983), and for the reproducible and sustained cell division of cotyledonary protoplasts of *Pinus pinaster* (David et al. 1984). Far less effort has been directed towards the study of the role of vitamins on plant development. Myo-inositol has received some attention. Jacquot (1966) found this vitamin to be advantageous in promoting bud formation in culture of *Ulmus campestris*. The involvement of myo-inositol in the synthesis of phospholipids and in cell wall pectins has been also confirmed (Anderson and Walter 1966).

It is a common feature in the tissue culture of conifers that the medium used to initiate shoots is unsuitable for the later stages of plant regeneration. Certainly, an understanding of the metabolic activity prior to and during the regeneration process is of vital importance in this respect. A pattern of heavy starch accumulation was observed in cultured cells of tobacco prior to the initiation of meristemoids and the formation of primordia (Thorpe and Murashige 1968). This finding reflects the need for a high content of carbohydrates in the medium especially of a relatively high

concentration of sugars. More recently Biondi and Thorpe (1982_b) reported a rapid depletion in lipids and free sugars by 10-fold and 6-fold respectively in the culture of radiata pine during the course of bud induction. These results, perhaps significantly, led them to the hypothesis that initiation of organized development *in vitro* involves a shift in metabolism (Obata-Sasamoto et al. 1984). In most cases, bud development and shoot elongation have been accomplished on a dilution of the initiation medium (1/3-1/2 strength of the mineral salt) with reduced sucrose level (1-2%) (e.g. Reilly and Brown 1976, Mehra-Palta et al. 1978, Jansson and Bornman 1980, Von Arnold and Eriksson 1981). According to Thorpe (1982) it is probable that inhibition of this process at a high level of carbohydrates is partly osmotic. In other cases, as with *Pinus radiata*, once the buds are induced a change of medium is necessary to allow for stem elongation (Horgan and Aitken 1981).

The nutrient media usually employed to induce roots on shoots obtained in conifers *in vitro* usually have a reduced mineral-salt content (1/8-1/2 strength of the mineral salt) with a lower level of sucrose (0.5-1%) e.g. *Thuja plicata* (Coleman and Thorpe 1977), *Pinus taeda* (Mott and Amerson 1981_a), *Pinus monticola* (Amerson and Mott 1982), *Picea mariana* & *Picea glauca* (Rumary and Thorpe 1984), *Pinus contorta* (Patel and Thorpe 1984). Part of the effect of these reductions in the constituents of the nutrient medium is to reduce the callusing at the base of the shoots, otherwise the callus may grow faster than the root primordia and suppress outgrowth of the roots (Sommer and Caldas 1981). In this connection, rooting of induced shoots on conifer explants has been attempted using other substrata

under non-sterile conditions. Such an approach avoids some of the problems associated with rooting in sterile agar. Roots induced in agar are often poorly developed, unbranched, and unable to survive transplantation from agar into soil, due to the poorly developed vascular connections between the root and shoot (Biondi and Thorpe 1982_a, Thorpe and Biondi 1984). It may be that the use of alternative substrata would be as effective as, or superior to, rooting in agar aseptically. Mehra-Palta et al. (1978) rooted shoots of *Pinus taeda* in a mixture of vermiculite-sand or peatmoss-sand (1.5:1) under mist (10 s at 6 min intervals) with bottom heat (26 °C) and a high light intensity. About 50% of the cultured shoots rooted and the roots formed in soil were more vigorous than those formed in agar. A higher rooting response of 86% has been reported with *Pinus radiata*. Here the shoots were pretreated for 5 days under non-sterile conditions on a water-agar medium containing a mixture of auxins, then transferred to a peat-pumice (1:1) rooting mixture and kept under mist during the first week (Horgan and Aitken 1981). Only few plantlets were obtained with *Picea abies* and *Pseudotsuga menziesii* using perlite as a rooting medium (Chalupa 1977_b), or with *Tsuga heterophylla* using a 'mica-peat' soil mixture (Cheng 1976). Limited success has been achieved with rooting *Pinus pinaster* shoots in a mixture of peat and perlite (1:3) (Rancillac et al. 1982).

1.3.2 The physical culture environment

The more variables in the culture environment that can influence growth and development are light and temperature. It is clear from a consideration of the literature that the physical aspects of the culture environment have not as yet been critically evaluated, and most

reports on the culture of conifers are deficient in this respect.

(i) *Physical properties of the nutrient medium*

For the successful regeneration of plants from culture the nutrient medium must be suitable both in terms of its chemical and physical properties (Murashige 1974). The physical form of the medium, i.e., whether it is a gel or a liquid, play an important role in growth and differentiation. Culture media are often in the form of a gel with agar ranging from 0.6-0.8% (e.g. Sommer et al. 1975, Bonga and McInnis 1975, Mott and Amerson 1981_a). Agar is an organic complex used as a support for tissues, but it also influences water potential and hydraulic and diffusive flow. Therefore, lowering the agar concentration or using liquid media may improve the availability of nutrients and hormones (e.g. Reilly and Brown 1976, Von Arnold and Eriksson 1979_b, Horgan and Aitken 1981). However, these attempts have posed new problems, particularly, the occurrence in many tissues of a condition known as 'vitrification' (Yeoman 1986). Vitrification or hyperhydric transformation is the phenomenon where leaves become translucent, appearing glassy or waterlogged (Debergh 1983). It is a physiological disorder associated with specific changes in the appearance of induced organs *in vitro*. (Kevers et al. 1984). Under similar conditions cultured Shoots of *Pinus brutia* turned bright green and their needles adhered to each other (Abdullah et al. 1985). Green, translucent, and watery looking buds appeared in *Pinus radiata* cultures (Horgan and Aitken 1981), and those of *Picea abies* (Von Arnold and Eriksson 1979_b). Vitrified shoots of *Pinus radiata* were difficult to root and may not survive transfer from sterile to non-sterile conditions (Horgan and Aitken 1981). Attempts have been made to

acclimatize vitrified shoots of *Picea abies* to ensure their subsequent survival in a greenhouse (Von Arnold and Eriksson 1984). Debergh (1983) postulated, from somewhat limited data, that an excess of cytokinins under inductive conditions coupled with a low agar concentration caused vitrification. Ethylene has also been implicated (Kevers et al. 1984). However, sometimes vitrification occurs in one laboratory and not in another, even when apparently the plant and the *in vitro* techniques are the same (Debergh 1983). In culture of *Pinus brutia*, vitrification was only detected after many subcultures (Abdullah et al. 1985).

The use of a stationary liquid medium may restrict the availability of oxygen for continuous growth of the cultured tissues. This may be overcome by agitation but this requires expensive and bulky shakers. Generally liquid media have been found to be less satisfactory for the induction of adventitious organs in conifers than agar media. Reilly and Brown (1976) reported that a low percentage of *Pinus radiata* needles produced buds in SH liquid medium compared with a much higher frequency of needles forming buds on an agar medium of the same composition. On the other hand, it has recently been claimed that the reliable production of buds from shoot tips of seedling of radiata pine was obtained using liquid SH medium as the initiation medium, though no comparative data were given using an agar based SH medium. Also 10% of the shoot tips incubated in the liquid turned completely red and failed to produce shoots (Horgan and Aitken 1981). Using a liquid rather than an agar medium did not enhance shoot formation and in some cases reduced it in *Picea glauca* and *Picea mariana* (Rumary and Thorpe 1984). Since agar is likely to influence the water potential and also perhaps

the availability of the nutrient components to the tissues, it would seem sensible to use a low concentration whenever possible. The ability to form adventitious buds on cultured explants of *Picea abies* increased as the agar concentration decreased until a stage was reached (0.3%) when the organ became completely submerged then the induction of buds tended to stop (Von Arnold and Eriksson 1979_b).

It is notable from the literature that the increased number of conifers regenerated *in vitro* throughout the last decade has been accomplished almost exclusively by using agar rather than liquid media. But agar-grown roots, have frequently exhibited undesirable characteristics which are thought less likely to occur with other substrata under non-sterile conditions (Thorpe and Biondi 1984).

(ii) Light

Light is one of the most important environmental factors. Photon flux density, photoperiod, and spectral composition all influence the extent and degree of differentiation in tissue cultures (Murashige 1974). Most researchers do not critically evaluate the light requirements for optimal organogenesis, but there are some reports that emphasize the importance of this factor. It is highly improbable that the role of light on shoot formation *in vitro* is primarily related to the supply of carbon from photosynthesis (Douglas et al. 1982), but is linked to a photomorphogenetic response (Murashige 1974, Hughes 1981). In a more recent study, the involvement of light and its interaction with applied cytokinins in the development of meristematic tissues and subsequent shoot formation has been emphasized (Villalobos et al. 1984_a). The daily light requirements of cultures of gymnosperms have rarely been systematically evaluated. A 16 h light /8 h dark

photoperiod resulted in a higher frequency of shoot formation than under continuous light in cultures of *Pinus contorta* (Patel and Thorpe 1984), *Picea abies* (Von Arnold 1982_b), and *Pinus rigida* (Patel et al. 1986). Also in other species the most commonly used photoperiod regime is a 16 h day vs. 8 h night (e.g. Bonga and McInnis 1975, Brown and Sommer 1977, Bonga 1977, Winton and Verhagen 1977, Mehra-Palta et al. 1978, Bornman and Jansson 1980, Aitken et al. 1981, Bonga 1981, Jelaska et al. 1982, Rancillac et al. 1982, Rumary and Thorpe 1984, Patel and Thorpe 1984).

The photon flux density used for the initiation stage in conifers ranges between 60-150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ e.g. *Pinus sylvestris* (Bornman and Jansson 1980), *Pinus taeda* (Mehra-Palta et al. 1978), with the great majority subjected to levels in about the middle of this range, i.e. 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ e.g. *Pinus resinosa* (Bonga and McInnis 1975), many conifers (Brown and Sommer 1977), *Pinus radiata* (Reilly and Washer 1977), *Pinus monticola* (Mott and Amerson 1981), *Pinus pinaster* (David et al. 1982), *Pinus rigida* (Patel et al. 1986). Analysis of the literature reveals that a lower light intensity, 35-70 $\mu\text{mol m}^{-2} \text{s}^{-1}$, has been found beneficial for the rooting of conifer shoots *in vitro* e.g. Coleman and Thorpe 1977, Mott and Amerson 1981_a, Rancillac et al. 1982, Rumary and Thorpe 1984, Patel and Thorpe 1984). According to Yamakawa et al. (1979) more than 90% of the IAA was decomposed by fluorescent light at about 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 7 days. However, during plantlet hardening, prior to planting out, it is desirable to subject the cultures to a higher light intensity (Thorpe and Biondi 1984). Full sunlight is as high as 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

(iii) Temperature

Temperature is also a key factor in controlling the growth of plants. Most cultured tissues grow well within the temperature range of 20-27 °C, however the maximum rate of propagation of a species is presumably temperature dependant (Murashige 1974). In a more recent study on *Pinus contorta in vitro*, it has been found that culturing embryos at 27 °C rather than 20 °C was beneficial in increasing the frequency of bud formation by about 17%, and also reducing the time taken for bud induction by 10 days (Patel and Thorpe 1984). Also, in *Pinus rigida*, the process of bud formation was enhanced from 20% to 90% by increasing the temperature regime from 20 °C to 27 °C (Patel et al. 1986). Except for these studies, culture temperature like the light regime has often been selected arbitrarily without experimental verification. Moreover, the temperature measured and quoted in publications is often that of the growth chamber rather than of the tissues inside the culture containers. For the initiation stage of conifer cultures, a constant temperature ranging between 20-29 °C has usually been employed e.g. *Pinus radiata* (Reilly and Brown 1976), *Pinus sylvestris* (Bornman and Jansson 1980), *Pinus nigra* (Jelaska et al. 1982), with the most common temperature being $21 \pm 1-3$ °C e.g. *Pinus resinosa* (Bonga and McInnis 1975), *Pinus monticola* (Mott and Amerson 1981), *Picea abies* (Jansson and Bornman 1981), *Abies balsamae* and *Pseudotsuga menziesii* (Bonga 1981). On the other hand, a relatively lower temperature, as low as 19 °C (Cheng and Voqoui 1977) and up to 25 °C (Horgan and Aitken 1981) has been found to be satisfactory for the rooting of conifers.

1.3.3 Influence of growth substances

(i) Induction and development of adventitious and axillary buds

Growth and morphogenesis are routinely accomplished by the incubation of cultured tissues on specific media containing growth substances which play a vital role in determining development. Auxins and cytokinins have been recognized as the primary morphogens in triggering morphogenesis (Skoog 1971, Fosket 1980, Thorpe 1980). Since the mid-seventies when the first regenerated plantlets of conifers were successfully obtained (Sommer et al. 1975), investigations in this field have been mainly directed toward manipulating the differentiation pattern of the cultured tissues by control of the applied auxin:cytokinin balance; as described by Skoog and Miller (1957). However, recent work on conifers confirms that the inclusion of auxin together with a cytokinin is unnecessary, since the cytokinin alone has been found to be sufficient to bring about shoot formation in cultures of many species e.g. *Picea abies* (Von Arnold and Eriksson 1979_a; Bornman 1983), *Pinus radiata* (Biondi and Thorpe 1982_a), *Pinus sylvestris* (Tranvan 1979; Bornman and Jansson 1980), *Pinus contorta* (Von Arnold and Eriksson 1981), *Pinus brutia* (Abdullah et al. 1985), *Pinus rigida* (Patel et al. 1986). The addition of auxin (NAA), even at low levels of 0.01-0.05 mg l⁻¹, has been shown to enhance more callus proliferation than organogenesis (Bornman 1983, Rumary and Thorpe 1984). The critical balance of growth substances must be within the tissues at organ-forming loci (Thorpe 1980, Bornman 1985). To achieve desired morphogenetic responses it would be therefore necessary to devise a precise ratio (endogenous and exogenous) of auxin:cytokinin (Fosket 1980). At the present time, however, information about the

levels of endogenous plant growth hormones, and how these levels change in specific tissues of the explant during differentiation is very difficult or impossible to acquire (Zaerr and Mapes 1982, Bornman 1985). It is vital in the first place to find the kind and quantity of growth substances necessary to supplement those already present in the explant.

Evidence emerging from the literature on conifers *in vitro* confirms the necessity of certain types of exogenous cytokinin, for individual species, such as BAP for *Picea sitchensis* (Webb and Street 1977); *Biota orientalis* (Thomas et al. 1977); *Pseudotsuga menziesii* (Cheng 1979); *Pinus sylvestris* (Bornman and Jansson 1980); and *Pinus rigida* (Patel et al. 1986), zeatin (ZEA) or BAP for *Pinus taeda* (Mehra-Palta et al. 1978), Kin for *Picea abies* (Von Arnold 1982). A given cytokinin may not work well in some species, but be quite effective in others, for instance 2iP was found to be most effective for shoot production in *Pinus radiata* (Reilly and Brown 1976); *Picea glauca* and *Picea mariana* (Rumary and Thorpe 1984). Generally, the efficiency of growth substances as morphogens depends on their availability which is also affected by concentration and stability in the medium during preparation, sterilization, and the culture period (Jacobsen 1983). BAP seems to have become the favourite cytokinin of tissue culturists in recent years. It is stable, inexpensive, readily available and most importantly it is highly effective. It seems that it may induce the production of natural growth substances such as zeatin within the tissues and thus work through a natural hormone system to induce organogenesis (Zaerr and Mapes 1982). Perhaps the early observation on the diverse morphogenic influences by different cytokinin types led

Cheng (1975), Webb & Street (1977) and later Von Arnold (1982_a) to raise the possibility of increasing the yield of shoot production by using a mixture of different cytokinins, though none of these workers furnished supporting data. Evidence has recently been produced to confirm the superiority of a mixture of BAP+2iP compared to either cytokinin alone in enhancing the production of shoots in cultures of *Picea glauca* and *Picea mariana* (Rumary and Thorpe 1984).

The level of growth substances required for the initiation stage of conifers *in vitro* seems to depend on the explant source used. A combination of BAP+NAA of $1+0.001 \text{ mg l}^{-1}$ respectively was sufficient for activation of axillary development on the excised shoot tips (1-1.5 cm length) from 20 day-old seedlings of *Pinus nigra* (Jelaska et al. 1982). Gupta and Durzan (1985) were able to achieve axillary shoot production on excised shoot pieces (3-4 cm length) from mature trees of *Pinus lambertiana* and *Pseudotsuga menziesii*, even without using any growth substances. By contrast, a higher concentration of BAP of 10 mg l^{-1} was required to stimulate axillary bud formation on embryonic material of *Pinus pinaster* (David et al. 1978). For *de novo* initiation of primordia on non-meristematic tissues of conifers, higher quantitative requirements has been demonstrated.

However, adventitious bud production on embryonic and cotyledonary culture systems has been accomplished by using relatively low levels of cytokinins (with or without auxin), i.e. 0.8 mg l^{-1} BAP with *Pinus pinaster* (David et al. 1982), $1-1.5 \text{ mg l}^{-1}$ BAP with *Pseudotsuga menziesii* (Cheng 1975); *Pinus contorta* (Webb and Street 1977); *Pinus taeda* (Mehra-Palta et al. 1978); *Pinus williciana* (Konar and Singh 1980), *Pinus strobus* (Minocha 1980), 3 mg l^{-1} BAP with *Pinus brutia*

(Abdullah et al. 1985), and up to 5 mg l^{-1} BAP with *Pinus radiata* (Reilly and Washer 1977); *Pinus sylvestris* (Tranvan 1979); and *Pinus rigida* (Patel et al. 1986), or 5 mg l^{-1} 2iP with *Picea abies* (Von Arnold and Eriksson 1978). Pines are xeromorphic trees with narrow leaves covered with a heavy cuticle, and a small surface/volume ratio with sclerenchyma (Mirov 1967), and it is possible that some of these attributes might impose physiological and morphological constraints on culture *in vitro* (Bornman 1983). Bud formation on needle culture has been reported using an especially high level of cytokinins ($5\text{-}10 \text{ mg l}^{-1}$ with or without auxin), e.g. *Pinus radiata* (Reilly and Brown 1976), *Picea abies* & *Pseudotsuga menziesii* (Chalupa 1977_b), *Pinus pinaster* (David et al. 1978); *Picea abies* (Jansson and Bornman 1980).

Recently Bornman (1983) presented a new technique for cytokinin application: the pulse treatment (application of 125 mg l^{-1} BAP with continuous shaking for 3 h) of embryos of *Picea abies*. According to Bornman (1983) this method proved more effective than the conventional method of incorporation of the cytokinins in the media; a higher number of adventitious buds were formed in a shorter time, however, he agreed that the uptake of cytokinin by explanted tissues is affected *inter alia* by the possible development of the outer cuticular layer. Lately, considerable attention has been given to the uptake of cytokinins by pulse-treated embryonic tissues of *Picea abies* (Vogelmann et al. 1984, Staden et al. 1986). Both of these studies confirmed that the amount of labelled cytokinin which entered *Picea* embryos was very low, and the pattern of uptake was consistent with the view that BAP entered the explants passively and that uptake into the explants may be restricted through microchannels in the cuticle.

(ii) Formation of shoots

in conifer *in vitro*

It has been demonstrated that shoot growth was fostered when the induced buds were excised and transferred onto a medium devoid of growth substances (e.g. Cheng 1975, Cheng 1976, Chalupa 1977_b, Reilly and Washer 1977, Konar and Singh 1980, Aitken et al. 1981). Bud development and shoot elongation have also been obtained by the inclusion of activated charcoal in the elongation medium e.g. Mehra-Palta et al. 1978, Boulay 1979_b, Von Arnold and Eriksson 1981, Mott and Amerson 1981_a, David et al. 1982, Bornman 1983, Patel and Thorpe 1984, Rumary and Thorpe 1984, Patel et al. 1986). The role of activated charcoal (AC) is as yet unclear; however, Weatherhead et al. (1978; 1979) and Misson (1982) subscribe to the idea that AC may absorb cations, excess growth substances, phenolics, and toxins created during autoclaving and culture. In fact, the need for more information about factors deterring buds from developing into distinct shoots is clear. According to Mott (1981) tissue culture procedures may inadvertently trigger some dormancy routine in many of the buds produced. In this connection, some reported data reveal a residual effect on shoot formation exerted by the high level of growth substances previously used in the initiation stage (e.g. Mehra-Palta et al. 1978, Amos and McCown 1981, Abdullah et al. 1985, Patel et al. 1986). Likewise, the formation of shoots from induced buds in cultures of *Pseudotsuga menziesii* (Cheng 1979) and *Picea abies* (Von Arnold 1982_a) has been shown to be affected by the type of cytokinin used for the initiation of these buds. Kinetin, for instance, stimulated the formation of significantly more needle primordia (Cheng 1979).

(iii) Induction of adventitious roots

The process of adventitious root formation can be divided into at least two stages: the initiation of root primordia following cutting and wounding and the stage of root emergence and growth. The importance of auxins in the regulation of root initiation has already been recognized (e.g. Scott 1972, Fosket 1980), and according to Haissige (1974) the auxin-phenol complex that is synthesized by polyphenol oxidase is critical for completion of this stage. The majority of papers that reported induction of root primordia in conifers *in vitro* used NAA ($0.1-1.0 \text{ mg l}^{-1}$) and/or IBA ($0.01-50 \text{ mg l}^{-1}$). In some species, it has been shown that maximum root primordia formation occurred in the presence of auxins and cytokinins. However, during the stage of root formation and development auxins are not required and the treated shoots have commonly been subcultured onto a medium without any growth substances. Progress in rooting *in vitro* has been good with a variety of dicotyledonous species, but much slower with conifers. At the present time, plantlets have been regenerated from 18 species, but success has been restricted to induced shoots from juvenile tissues, and the frequency of shoots forming roots varied widely among species. In some species, the success is limited to a few rooted shoots: rooting of *Pinus palustris* (no data were given) was obtained with 10 mg l^{-1} IBA for 1 month (Sommer et al. 1975); and shoots of *Biota orientalis* treated with NAA (1 mg l^{-1}) in conjunction with either ZEA or Kin (1 mg l^{-1} for each) proliferated callus-bearing root-like structures (Thomas et al. 1977); while Minocha (1980) found that after 10-12 weeks incubation on a medium including IBA at $0.01-2.0 \text{ mg l}^{-1}$, a few shoots of *Pinus strobus* produced 1 or 2 roots. A low frequency of rooting (<40%) has been reported for other species:

spontaneous root formation (20%) occurred in shoots of *Picea sitchensis*, but shoots initiated on 2iP and Kin have a higher frequency of root induction than those induced on BAP (Webb and Street 1977). In *Picea abies*, however, root induction (33%) was enhanced by infusion of IBA (5 mg l^{-1}) into the explants (Bornman 1983); and a mixture of IBA+NAA at concentration of $5+1 \text{ mg l}^{-1}$ for 5 days gave rise to 37% rooted shoots in *Pinus rigida* (Patel et al. 1986). Relatively higher percentages (<70%) of shoots forming roots have been achieved for a few other species: 50% of the shoots that were induced on cotyledon explants of *Thuja plicata* formed roots with 50 mg l^{-1} IBA (for 4-8 weeks incubation), but in shoots induced on explants of 4-10 year-old trees, only 11% did so (Coleman and Thorpe 1977). A pulse treatment of adventitious shoots of *Pinus monticola* for 7 days with a combination of BAP+NAA of 0.1 mg l^{-1} for each provided 64% rooting (Amerson and Mott 1982); and with *Pinus contorta*, shoots treated with rooting powder containing 0.1 mg l^{-1} IBA resulted in 52% rooting (Patel and Thorpe 1984). Direct root formation has been reported with *in vitro* shoots of *Tsuga heterophylla*, however, no data were furnished (Cheng 1976).

A substantial rooting of 75-100% has only been attained in 8 conifer species: in *Pseudotsuga menziesii*, a combination of 2iP+NAA (0.5 mg l^{-1} for each) elicited maximum rooting of 82% (Cheng 1979); while soaking of induced shoots of *Sequoia sempervirans* for 24 h in an IBA-fungicide solution gave rise to subsequent rooting of 80% (Boulay 1979_b); in *Pinus taeda*, treated shoots with a mixture of NAA+BAP of $0.5+0.1 \text{ mg l}^{-1}$ rooted with 75% success (Mott and Amerson 1981_a); and a substantial rooting of 80-100% has been obtained in *Pinus pinaster* by treating the axillary shoots with NAA at a concentration of 1 mg l^{-1} for 12 days

(Rancillac et al. 1982); incubation of shoots of *Pinus radiata* on a mixture of NAA+IBA of $0.5+2.0 \text{ mg l}^{-1}$ for 5 days caused rooting of 86% (Aitken et al. 1981, Horgan and Aitken 1981). More recently, charcoal was also found to enhance rooting in some species: Rumary and Thorpe (1984) reported a high frequency of rooting, 83% for *Picea mariana* and 80% for *Picea glauca*, by dipping the induced shoots into a rooting powder containing 0.1 mg l^{-1} IBA and maintaining the treated shoots in a medium including 0.2% AC; also 75% rooting of hypocotyl cuttings from 20 day-old seedlings of *Pinus sylvestris* has been achieved when these cuttings were maintained in a medium containing $0.1-20 \text{ g l}^{-1}$ AC (Gronroos and Von Arnold 1985).

Buds and shoots can be induced on explants of mature trees of some conifers, but rooting is often the limiting step e.g. *Pinus taeda* (Mehra-Palta et al. 1978), *Picea abies* (Von Arnold 1979), *Pinus pinaster* (David 1979), *abies balsamae* (Bonga 1981), *Pinus sylvestris* (Thompson and Zaerr 1982), *Larix decidua* (Bonga 1984), *Pinus lambertiana* & *Pseudotsuga menziesii* (Gupta and Durzan 1985). However, rooting of induced shoots from explanted stump sprouts of 50 year-old trees of *Sequoia sempervirens* has been reported (Boulay 1979_b).

(iv) Development of adventitious root

system and mycorrhizae formation

A critical review of the literature reveals that only few deal with survival and performance of plantlets produced *in vitro* after transplanting from sterile condition into soil (e.g. *Pinus radiata* (Aitken and Gleed 1984; Smith 1986), *Pseudotsuga menziesii* (Timmis and Ritchie), *Pinus taeda* (McKeand and Allen 1984; McKeand and Frampton 1984). Rooting is a slow and most difficult step in micropropagation

of conifers. Perhaps this could partially account for the fact that most shoots *in vitro* produce roots indirectly via callus tissues. Excess callus proliferation can disturb the formation vascular connections between stem and root and may bring about nutritional disorders (Biondi and Thorpe 1982_a, Rancillac et al. 1982). An anatomical study of the developmental pattern of adventitious root formation *in vitro* in hypocotyl cuttings of 20-day-old seedlings of *Pinus sylvestris*, showed that a vascular connection between the developed root and the hypocotyl was always present, however, some cuttings which developed large wound tissues failed to root. But even if roots developed from such a large complex of tissues, they had abnormal spirally oriented vascular tissues at their basal parts (Gronroos and Von Arnold 1985). Virtually, the success of transplanting and survival of the plantlets will depend on the quality of the induced root system and the latter is affected by the rooting treatments (Rancillac et al. 1982). No attention has as yet been paid to the carry-over effects of growth substances, generally used for the initiation stage, on root quality and subsequent vigour and performance of the rooted shoots *in vivo*. The establishment of an effective and uniform root system on shoots grown *in vitro* is critical to the production of mass clonal material for commercial purposes (Mott 1981, Sommer and Caldas 1981).

Certain trees such as *Pinus* species will not grow and develop normally without being mycorrhizal (Harley and Smith 1983). The feeder roots of pine trees are associated with highly specific root inhabiting fungi, in which these infected feeder roots are called mycorrhiza (fungus-root). It has been generally accepted (e.g. Bjorkman 1970,

Hacskeylo 1973, Harley and Smith 1983, Dixon and Marx 1984) that the relationship between these fungi and the plants is symbiotic, since the infected plants have generally been shown to exhibit an improved nutrient uptake and increased tolerance to adverse environmental extremes, whilst the fungus depends on the host plant for nutrient supply (carbohydrates) for growth and development. Many areas of applied and basic research on mycorrhizal symbiosis remain fertile for exploration. In micropropagation the importance of the association is in the establishment phase (Dixon and Marx 1984). From the few published reports it has been recognized that before the potential for mycorrhizogenic formation by *Pinus pinaster* plantlets can be expressed, a certain age or degree of 'maturation' of the parent root tissue is required (Faye et al. 1980). Mycorrhizal branching was obtained when the roots were induced with low level (1.0 mg l^{-1}) of NAA (Rancillac et al. 1982). Also Gay (1982) confirmed that rooting in cuttings of *Pinus halepensis* was significantly increased in the presence of certain ectomycorrhizae (*Hebeloma biemale*) and low levels ($0.75\text{-}1.0 \text{ mg l}^{-1}$) of NAA and IBA, and the roots formed were covered with a fungal sheath displaying ectomycorrhizal short roots with a characteristic dichotomous branching.

1.3.4 Selection of explants

Although any plant part can be used as the source of explant, the choice is usually made so as to maximize the release of morphogenetic potential. According to Murashige (1974), in deciding on a suitable explant, the investigator should consider (a) the organ that serves as the tissue source, (b) the physiological and ontogenetic age of the organ, (c) the season in which the explant is obtained, (d) the size of the explant, and (e) the overall quality of the plant from which the explants are to be obtained. Using juvenile tissue such as embryos and seedling parts for the induction of adventitious buds seems to be the most successful method today in the mass propagation of conifers *in vitro*. In fact, the use of juvenile tissues of conifers has a potential application for the *in vitro* regeneration of plants from scarce and costly seeds derived from controlled pollination, or where a clonal alternative to reforestation with seedlings is desired (Libby 1979, Bornman 1983). In developing a procedure for commercial use, however, selection of the explant source should be carried out systematically and deliberately (Murashige 1974). One of the best examples is the work done with micropropagation of *Pinus radiata* with excised cotyledons from about 1 week-old germinated seeds (Aitken et al. 1981, Horgan and Aitken 1981). In New Zealand, using this explant, it was possible to generate 16700 shoots per 100 seeds of this species, with a maximum shoot production of 257 shoots per clone within 6 months (Smith 1986). Accordingly, up to 180 rooted shoots per seed were obtained within a further 12 weeks (Thorpe 1985). In *Pinus taeda*, a production of 10-100 plantlets per cultured cotyledon, excised from partially germinated seeds, was achieved within 6.5 months (Mott and

Amerson 1981_a). In a comparative study of shoot production in seedling tissue of black and white spruce, it was found that 25 to 28 day-old epicotyls, consisting of intact cotyledons and a 2 mm hypocotyl stub for black spruce, and trimmed cotyledons (ca. 1 mm removed) with no hypocotyl tissue for white spruce, were the best explants. An average production of 40 plantlets has been recorded for each species within 6.5 months (Rumary and Thorpe 1984). In all these cases, the overall efficiency seemed to depend on sustained activity of the induced meristems to form buds and shoots over a long time, though high rooting is also required. In *Picea abies* for instance, it was easy to obtain a good yield of shoots (3-150 shoots per embryo with an average of 10-20 shoots (Von Arnold and Eriksson 1986), but the problem was to regenerate plantlets from these buds (Von Arnold 1982_b, Bornman 1983). In some other species low rates of shoot production have been recorded: 37 shoots (19 plantlets) per embryo was obtained in cultures of *Pinus contorta* within 160 days (Patel and Thorpe 1984), 19 shoots and 7 plantlets per *Pinus rigida* embryo were achieved within 14 and 24 weeks respectively (Patel et al. 1986), while in cultures of *Pinus monticola*, an average of 17 buds with a range of 1-112 buds per embryo has been recorded (Mott and Amerson 1981_b). No quantitative data were reported for the number of shoots and plantlets per cultured explant with other conifers e.g. embryos of *Pinus palustris* (Sommer et al. 1975), *Tsuga heterophylla* (Cheng 1976), *Picea sitchensis* (Webb and Street 1977), *Thuja plicata* (Coleman and Thorpe 1977), *Pseudotsuga menziesii* (Cheng 1979), *Pinus sylvestris* (Bornman and Jansson 1980).

The micropropagation of conifers via callus culture systems has been far less successful, and in the best cases only a few shoots (e.g.

^u*Sequoia sempervirens* (Ball 1950), *Pseudotsuga menziesii* (Winton and Verhagen 1977), *Pinus williciana* (Konar and Singh 1980)) or individual plantlets have been recorded (e.g. *Pinus palustris* (Minocha 1980), *Larix decidua* (Bonga 1984)).

It is claimed that plants regenerated directly from meristematic explants have a higher degree of chromosome stability than those obtained via callus or suspension cultures (Hussey 1978, Vasil and Vasil 1980, Sharp and Evans 1981), and in those cases where the regenerated plants show signs of chromosome instability such as polyploidy, the most obvious factor was the source of explant or its culture history (Hakman et al. 1984). So far some of the published reports have revealed that cells from shoots, or root tip cells from adventitious plantlets of *Picea abies* (Von Arnold 1982_b; Hakman et al. 1984), *Pinus taeda* (Mott et al. 1977; Renfroe and Berlyn 1984) *Pinus nigra* (Papes et al. 1983), and *Pinus contorta* (Patel and Thorpe 1984) usually had a normal diploid component of chromosomes. However, in *Pinus taeda* polyploidy and mixoploidy occurred in the adventitious buds, but with proper attention to the levels of growth substances and timely transfer between media the problem could be minimised (Mott et al. 1977). On the other hand, Patel and Berlyn (1982) found that in *Pinus coultri* after 6 weeks in culture, about 80% of the nuclei of induced adventitious buds had a DNA content greater than 4n. They suggested that the exogenously supplied cytokinin could have induced instability of the tissue when cultured *in vitro*. Also, in cultures of *Pinus taeda* the genetic stability deteriorated to some extent if auxin was added or when callus was induced (Renfroe and Berlyn 1984). Furthermore, changes in chromosome volume and DNA content may occur even when the

chromosome number is unaffected (Berlyn et al. 1986).

Regeneration *in vitro* via organised systems, i.e. preformed meristems, has been put forward as a practical solution to the cloning of forest trees. However, only limited progress has been made in this area. The proliferation of axillary buds has been accomplished in resting, primary meristematic cells located in the axils of the cotyledons and juvenile needles or at the apex of brachyblasts. The growth of these meristems is often inhibited by the activity of the main shoot, because of apical dominance (Kramer and Kozlowski 1979). One of the key limiting factors is the supply of cytokinin, since these meristems, under apical dominance, are supposed to lack the ability to synthesize their own cytokinins to continue development (Wickson and Thimann 1958). Successful examples of axillary bud production on juvenile tissues include few conifers. Axillary bud proliferation has been obtained on cotyledons and hypocotyls of *Pinus pinaster* but the process seemed to depend on the presence of the cotyledons (David et al. 1978). In *Pinus nigra*, axillary bud development has been reported on explanted hypocotyls (1-1.5 cm) from 20 day-old seedlings. However, the buds failed to develop into shoots and a poor survival rate was recorded (Jelaska et al 1982). Boulay (1976) reported the production of 3-10 axillary buds for each cultured shoot tip of *Pseudotsuga menziesii* from seedlings under 2 year-old. Because each axillary shoot can in turn produce its own axillaries and so on, a potentially unlimited system of proliferation is expected in almost all species which possess axillary meristems. In *Pinus pinaster*, for bud multiplication, when each new bud had elongated into a shoot stem with needles, they were exposed to cytokinin to induce additional axillary

buds (David 1979). In Douglas fir, the developed axillary buds were excised and transferred to a medium containing activated charcoal that promoted extension, and the shoots were then subdivided and returned to the multiplication medium with added cytokinin (Boulay 1979).

However, great difficulty has been experienced particularly when explants are taken from mature trees. This is attributed partially to the high polyphenolic content in the tissues of many woody species e.g. *Pinus sylvestris* (Von Arnold 1979), *Tectona grandis* (Gupta et al. 1980), date palm (Tisserat 1984), *Picea abies* (Von Arnold and Eriksson 1986). The development of brown pigmentation is presumably due to the accumulation of tannins and other phenolic compounds via the action of the enzyme polyphenol oxidase, which occurs when tissues are wounded (Smith 1968). The activity of the polyphenol oxidase was found to depend on unspecific binding of copper to this enzyme which could be removed by treatment with chelating agents (Lerch 1981). Earlier, Reinert and White (1956) had reported a similar finding with callus cultures of *Picea glauca*, and this has been confirmed by Durzan et al. (1973). It has been assumed that the chelating agents act by chelating the copper required for the activity of polyphenol oxidase, thus inhibiting their activities; or it may prevent synthesis of new oxidase by competing for copper (Smith 1968, Lerch 1981). According to Loomis and Battails (1966), and Goldstein and Swain (1965), the toxicity is probably mainly due to reversible hydrogen bonding to proteins. However, polymers such as insoluble PVP are capable of splitting phenol-protein complexes and thus restoring enzyme activity. Recently, Gupta et al. (1980) reported that browning was reduced and multiple shoots were formed when insoluble PVP (1%) was included in the culture

media of excised terminal buds of 100 year-old trees of *Tectona grandis*. However, to reduce browning in cultures of the date palm, activated charcoal was included in the culture medium with beneficial results (Tisserat 1984).

A further factor influencing the extent of browning can be the time of the year at which tissues are taken. In cultured shoot tips of *Hamamelis*, the browning intensity was found to be highest in explants sampled in May and was less in those collected during July and August (Christiansen and Fonnesbech 1975). Also, tissue browning was less of a problem and growth more rapid when shoot tips of apple were collected in mid-spring and summer than those collected in autumn and winter (Hutchenson 1982). The season in which explants are obtained may also influence the regenerative characteristics of the tissues in culture. It has commonly been observed that bud explants of conifers are more responsive to culture when they are just beginning active growth (before bud break) e.g. *Pseudotsuga menziesii* (Boulay 1979a), *Abies balsamae* (Bonga 1981), *Picea sitchensis* (Selby and Harvey 1985). Anatomical observations on conifers *in vitro* suggest that the variation in regenerative characteristics among explants depends on physiological age and the extent of differentiation among their constituent cells (Yeung et al. 1981, David et al. 1982, David 1982, Bornman 1983). The physiological state of the plant material can be modified *via* culture practices. Soaking explants from physiologically mature parts of the tree in water, or other solutions, has been shown not only to be effective in preventing or removing damaging phenolics, but also in leaching a factor inhibiting growth even in the absence of browning. Bonga (1977) found that if embryonic shoots (dormant buds minus scales)

of 15-20 year-old *Abies balsamae* were soaked either for 24 h in distilled water or for 15 min in a solution of 100 mg l^{-1} caffeic acid, bud formation occurred later upon transfer to a nutrient medium. Treatment of the buds with 0.1% malonic acid for 15 min after soaking, improved the response (Bonga 1981). In contrast, Von Arnold and Eriksson (1979_b) reported that soaking of naked buds of 5 to 75 year-old trees of *Picea abies* in water, BAP or IBA solutions did not stimulate organogenesis.

Difficulties encountered in the micropropagation of explants from mature trees may also depend on the quiescent state of the meristems used. In *Pinus pinaster*, bud development can be activated from the apical dome located between the needles of brachyblasts collected from 3-11 year-old trees (David et al. 1978). These were elongated and then promoted to form additional shoots by a cytokinin treatment (David 1979). Mehra-Palta et al. (1978) was able to stimulate the development of dormant meristems from the base of 80% of needle fascicles of 5 year-old greenhouse-grown seedlings of *Pinus taeda*. More recently, axillary bud proliferation was obtained on excised shoot pieces of 20 to 60 year-old trees of Douglas fir and 8 to 30 year-old trees of Sugar pine on media which included activated charcoal. Approximately 100 shoots could be obtained from each piece in a year (Gupta and Durzan 1985).

1.3.5 Regulation of the development process

Anatomical and physiological considerations

Anatomical studies of organogenesis *in vitro* provide insight into the nature of activation e.g. *Pinus radiata* (Reilly and Brown 1976; Yeung et al. 1981), *Pseudotsuga menziesii* (Cheah and Cheng 1978), *Pinus taeda* (Mott et al. 1977), *Pinus sylvestris* (Tranvan 1979), *Picea abies* (Jansson and Bornman 1981; Bornman 1983), *Pinus pinaster* (David et al. 1982), and *Pinus brutia* (Abdullah et al. 1985). In all cases, the process involves the dedifferentiation of certain cells in the original explant. This begins shortly after the isolation and culture of the tissues, with an acceleration in cell division and a consequent formation of meristematic zones or meristemoids. Apparently a specific type of cell division provokes the formation of *de novo* meristems. These meristemoids consist of a mass of small and isodiametric cells with prominent nuclei, densely staining cytoplasm, and microvacuolation. They are 'plastic' and in the meristematic state they readily respond to signals for *de novo* organ formation. Subsequently, the primordia arise from these meristemoids, and by further meristematic activity, the bud primordia then develop into adventitious buds with a shoot apex surrounded by a pair of needles. Accordingly, the formation of a new meristem has generally been accepted as a prerequisite for *in vitro* shoot regeneration (Thorpe 1980, David 1982). Detailed studies on the formation of meristematic activity in cultures of *Pinus radiata* revealed that cytokinins must be present during the first 3 days in the culture for any shoot formation to occur, but it is needed for 21 days for optimum shoot formation (Biondi and Thorpe 1982, Villalobos et al. 1984).

Whether each meristemoid begins from the division of a single cell or from many cells has not been determined. However, it is evident from the literature of conifer organogenesis that only certain cells in the cultured explant respond. According to Yeung et al. (1981) and David et al. (1982), adventitious budding depends mainly on the degree of cell differentiation, when cellular layers with a high mitotic activity, are relatively undifferentiated (David 1982, Bonga 1982_b), and not beyond the point of irreversibility (Jansson and Bornman 1981, Aitken et al. 1982, Bornman 1983), and, under the influence of exogenous cytokinins, these tissues were stimulated to form meristematic tissue. Therefore, the tissue responses of explant types (embryos, cotyledons, or needles) of conifers have been shown to be strongly correlated with their anatomy. The adventitious buds in both the embryo and cotyledon culture systems (e.g. Mott et al. 1977, Cheah and Cheng 1978, Von Arnold and Eriksson 1981, Tranvan 1979, Abdullah et al. 1985) originate from cells localized in the outer layers (hypodermis and epidermis). In scale leaves and needles, we have well differentiated epidermis and subepidermal layers and the underlying cells are already differentiated into the spongy mesophyll (Mirov 1967, Mott et al. 1977). Initiation of buds has mostly been reported in the less-completely differentiated mesophyll region at the needle base, the zone of elongation (Reilly and Brown 1976, Mott et al. 1977, David et al. 1982). In *Picea abies*, however, division giving rise to adventitious primordia was observed in epi- and hypodermal cells in younger needles, before they commence differentiation (Jansson and Bornman 1981), or else it was from the mesophyll layer (Bornman 1983).

It has been suggested that the developmental path might be made

shorter by bypassing the stage of *de novo* meristem initiation, and starting with plant material with pre-existing meristems (Murashige 1974). Unfortunately, little is known about axillary bud anatomy and physiology. Some time ago, Borthwick (1899) reported that unlike most of the conifers, juvenile pine normally bears an axillary bud at each scale leaf on the shoot axis and most of these axillary buds can develop into needle-bearing short shoots. Similar observations have been made by others (Stone and Stone 1943, Fink 1984). However, Fink reported that in at least some conifers (*Taxus baccata*, *Sequoia sempervirens*, *Cryptomeria japonica* and *Thuja occidentalis*) most leaf axils of the mature shoot still have the potential to form buds. So far, plant regeneration through the development of axillary buds has proved difficult with conifers, except with *Pseudotsuga menziesii* (Boulay 1976; 1979), *Abies balsamae* (Bonga 1977), *Pinus nigra* (Jelaska et al. 1982), and *Pinus pinaster* (David et al. 1978, David 1979). However, neither of these reports provided the important anatomical information about the origin of the buds produced.

Despite the great attention that has been given to aspects of the control and expression of organogenesis *in vitro*, we still do not understand how these processes are regulated. In fact, the search for triggers and markers of morphogenesis proceeds slowly, and an understanding of the underlying control of differentiation is still a long way off (Yeoman 1986). It seems evident from the literature that growth substances play a key role in determining the course of morphogenesis in higher plants (Thorpe 1980, Zaerr and Mapes 1982). So far the classic view of Skoog and Miller (1957), that the quantitative interactions between growth substances and other factors govern

development, has been accepted as a valid explanation. It seems from the literature that nearly all research carried out on conifers *in vitro* has been based on the quantitative manipulation of the exogenous growth substances which have been applied. However, the mechanism by which growth substances act at the cellular level remains to be determined and even the measurement of the endogenous levels of hormones in the tissue is still technically impossible (Skoog 1971, Thorpe 1980, Zaerr and Mapes 1982, Bornman 1985). An understanding of some of the events that lead to morphogenesis in tissue culture depends upon a better knowledge of the mode of entry, metabolism and subsequent distribution of the growth substances used. Accordingly, current attention has been concentrated in this direction, to investigate how much cytokinin was taken up by explants of *Picea abies* and *Pinus sylvestris* which were given short saturating doses ('pulse treatment'). Voglemann et al. (1984) and Von Arnold and Eriksson (1985) found that only about one third of the amount of growth substances in the medium entered the explants via a passive mode of uptake. Villalobos et al. (1984_b) found that CK-induced changes in the developmental pathways began within 24 h in culture of *Pinus radiata*. However, macromolecular synthesis (RNA and proteins) preceded CK-induced bud formation and was more restricted to the site where differentiation occurred. More recently, the metabolism of CK by excised embryos of *Picea abies* was studied by Staden et al. (1986) who stated that the major metabolite resulting from the cytokinin-pulse was a 'nucleotide' with biological activity, they postulated that the action of the cytokinin may be mediated via this nucleotide.

There has been a growing debate about the concept that auxins and

cytokinins and other combinations of growth substances are the only morphogens in triggering morphogenesis (Durzan 1984_b, Bornman 1985). Indeed, the classic view of Skoog and Miller (1957) has recently been challenged by Trewavas (1982, 1983), who proposed that changes in the sensitivity of tissues to growth substances is the critical factor in the control of plant development. This concept has been accepted by others, although, the term sensitivity is not often used in the literature. It has been accepted (Thorpe 1980, Yeung et al. 1981, David 1982, Bornman 1985) that only certain cells in a culture are susceptible or receptive to applied cytokinins. Also the sensitivity of cells and tissues in juvenile and mature explants to exogenous growth substances requires further definition and characterization, and the problem is that the cells and tissues under investigation are initially highly differentiated and developmentally heterogenous and show differential sensitivities to the imposed factors (Durzan 1984_a). Since the concept of 'sensitivity changes' is in itself too vague, future work should be directed to understanding possible mechanisms which could influence the response of cells to growth substances (Firn 1986). Coupled with the question of totipotency and dedifferentiation is the nature of cell activation, which starts a cell on its altered pathway toward dedifferentiation. The need for more information about the nature of the signal that leads to the activation of a cell is clear (Thorpe 1980). In other words, does a bidirectional transfer of materials, and also of information, occur during the course of development? Jacobsen (1983) concluded that it can be argued that morphogenetic activities of growth substances depend on the specific recognition of the morphogenic information carried by the plant hormone

molecule. In an attempt to describe a possible mechanism for whole-plant morphogenesis, Zajaczkowski et al. (1983) suggested that a higher degree of supercellular morphogenetic information than that obtained by mere hormone gradients seems to be required.

Again however, the organogenetic potential is unlikely to be released by any single exogenous factor or condition, for a multiplicity of interacting factors and conditions enter into the successful realization of this process (Steward 1983). In a few cases the interaction between exogenous factors has been investigated. Recently, the anatomical examination of *Pinus radiata* cotyledons *in vitro*, confirmed the morphogenetic interactions of light and cytokinin in shoot formation, and the data suggest that cytokinin is directly involved in the induction of shoot formation and both light and cytokinin are required for the development of meristematic tissues and subsequent shoot formation (Villalobos et al. 1984). Light is a major factor of the culture environment and has been shown clearly to have a photomorphogenetic effect *in vitro* in *Pinus brutia* (Abdullah et al. 1985) and *Pinus rigida* (Patel et al. 1986).

An important consideration still requiring attention is the effect of nutrients on levels of endogenous growth substances (Zaerr and Mapes 1982) and the possible interaction between the exogenous cytokinin and the nutrient medium (Mott and Amerson 1984). Apparently, endogenous gradients of hormones and nutrients, moving out of the medium and perhaps from the surrounding cells into the activated cells, may bring about the critical conditions that induce morphogenesis (Thorpe 1980, Zaerr and Mapes 1982, Staden et al. 1986). Currently, the importance of factors such as nutrients, physical properties of the culture medium

and the temperature has been well recognized (see Reilly and Brown 1976, David et al. 1978, Von Arnold and Eriksson 1979_b, Bornman 1983, Debergh 1983, Patel and Thorpe 1984, Rumary and Thorpe 1984, Villalobos et al. 1984_a, Von Arnold 1984, Abdullah et al. 1985).

Explant source is one of the first practical considerations in the application of cell and tissue culture technology to any crop plant, and with woody perennial crops a major problem has been the residual memory that the explant seems to have when isolated aseptically and forced to grow *in vitro* (Durzan 1984_a; 1984_b). It seems necessary therefore to consider the physiological condition of the explants and the degree of anatomical specialization of their tissues, since these vary according to the chronological age of the donor plant (Bonga 1982_a; 1982_b, Bornman 1985). For this reason, a more practical approach is necessary to control morphogenesis *in vitro* in order to regenerate plants with the genetic, developmental and physiological state appropriate to each particular application (Yeoman 1986). We probably have to learn much more about the metabolic and genetic switches that control development, before we can expect to discover how to unlock the regenerative potential of cells and tissues (Bonga 1982_a).

1.3.6 In vivo performance of tissue culture plantlets

Relatively little information about the *in vivo* performance and quality of conifer plantlets is available, and the limited data published relate to only three species (e.g. *Pinus radiata*, *Pinus taeda* and *Pseudotsuga menziesii*). Numerous differences between the growth pattern of the plantlets and the seedlings have been recorded, and generally the growth is less in the plantlets than in the seedlings. In *Pinus taeda*, shoot growth of the plantlets (measured as length) was roughly half that of the seedlings and the mortality in the former was higher during the 20 weeks of the experiment (Leach 1979). According to one recent published report, seedlings of this species grow to a greater size than the plantlets during 20 weeks incubation in the greenhouse; however, the relative rates of growth were approximately equal (McKeand and Allen 1984). Also in Douglas fir plantlets, the growth increment was less than that of the seedlings (Timmis and Ritchie 1984). Many of these differences between the plant type seemed to be related to the morphological differences between the root systems. Usually, good stem taper indicates favourable root development and balance between the shoot and the root. But the plantlets were found to have fewer lateral roots (thick and unbranched roots) than the seedlings, and the plantlet root system was therefore less effective than the seedlings at nutrient uptake for a given dry weight of root (McKeand and Allen 1984, McKeand and Frampton 1984). Moreover, Timmis and Ritchie (1984), found that about two thirds of the plantlets of Douglas fir began to exhibit some degree of plagiotropic growth immediately after rooting, and only 17% of the shoots of the culture produced vigorous upright plantlets within a year of transfer

into soil. Plantlets of *Pinus taeda* also tended to grow plagiotropically, but seedlings did not and the tendency was increased when plants were grown in smaller containers (McKeand and Frampton 1984). According to the authors, the poor quality of roots and the inactive meristems of the shoots, as well as the shoot/root interaction, are probably the main cause of this abnormality. Plagiotropism is the failure of the vegetative structure to assume tree form, and the lateral habit of a branch is observed. However, this phenomenon has been shown to be more persistent in propagules from old trees (Mott 1981, Bonga 1982_a). In this connection, an early appearance of fascicles on plantlets of *Pinus radiata* (Aitken and Gleed 1984) and *Pinus taeda* (Leach 1979; McKeand and Frampton 1984) have been reported, implying advanced maturation.

In all these cases, since both the shoots and the roots are adventitious in origin, a different pattern of development may be expected for these plantlets in comparison to the seedlings (McKeand and Frampton 1984). The need for more research to determine the specific cause for the abnormalities and variations in culture seems clear; though, some of the variations can be related to the choice of the explant over the life cycle of the tree (Durzan 1984_a, Timmis and Ritchie 1984).

Variability among trees is a major problem in forestry, and vegetative reproduction will greatly help to overcome this difficulty (Zobel and Talbert 1984). Indeed, the elimination of genetic variation among cloned plantlets is expected to increase uniformity and ease stand management (McKeand and Weir 1984). Genetic variation in somatic cells grown *in vitro* is termed 'somaclonal variation'. But any

variation must be of proven value or it may be regarded as unwanted 'somaclonal aberration' (Durzan 1984_b). Somaclonal variation is viewed in part as a differential expression and aberration of totipotency under *in vitro* conditions, and much of the genetic variation appears to arise from the use of synthetic growth substances (Durzan 1984_b). However, the importance of these changes for forest trees remains to be established (Durzan 1985, Yeoman 1986). According to Zobel and Talbert (1984) and Hasnain and Cheliak (1986), the potential for improving forests by exploiting the non-additive gene effects can exceed 50%, and this gain can only be exploited through cloning via tissue culture. Currently, some characteristics of *Pinus taeda* plantlets such as the lower incidence of infection by fusiform rust, compared to seedlings, has been seen to be beneficial (McKeand and Frampton 1984). Similarly, in *Pinus radiata*, 'Taradale' was introduced as a new micropropagule which has good potential as a shelterbelt tree (Aitken and Gleed 1984).

So far only the juvenile pathway of tissue culture has been demonstrated for these species, but the information gained on the micropropagation of these tissues, may be seen as a tool to establish methods for propagation of explants from mature trees. In *Pinus radiata*, the combination of micropropagation and cold storage may overcome problems associated with maturation. Accordingly, juvenile *radiata* pine clones (induced shoots) could be stored at 4° C whilst the same clones are being evaluated in field tests. Later, after 6-8 years the best clones could be propagated from juvenile shoots removed from their cold store. This approach may avoid the problems associated with the propagation of trees 6-8 years old as it should provide juvenile planting stock of the superior clones (Thorpe 1985). So far, in New

Zealand, radiata pine shoots have been cold-stored for 4.5 years, although plantlets have only been formed from shoots stored for 17 months (Aitken and Gleed 1984).

1.3.7 Some economic considerations

Using current methods, plantlets produced by tissue culture are expensive because of the multiple handling that is now required. Even with the hitherto high multiplication rate, it appears that micropropagated radiata pine will still cost at least NZ\$300 per thousand plantlets compared with a cost of NZ\$45 for a thousand seedlings (Smith 1986). In the USA, corresponding costs of US\$400 and US\$20 per thousand plantlets and seedlings respectively have been reported (McKeand and Weir 1984). Despite 11 years and 5 million dollars worth of research, no economical process has been achieved for the vegetative propagation of Douglas fir via tissue culture, though the search continues (Timmis and Ritchie 1984). In Canada, where the survival of seedlings is particularly low because of an adverse climate (Hasnain and Cheliak 1986), micropropagation is particularly attractive economically: Douglas fir seedlings cost 200-250 CAN\$ per a thousand whereas micropropagated stocks are 500-650 CAN\$ per a thousand.

1.4 STATEMENT OF OBJECTIVES

In this study, attention has been directed towards the development of an effective and reproducible method for vegetative propagation of *Pinus brutia* via tissue culture techniques. In the context of the forest industry, the prime objective was to achieve an alternative method of tree improvement via mass *in vitro* propagation of preferred genotypes.

CHAPTER 2

MATERIALS AND METHODS

- 2.1. Plant material
- 2.2. Nutrient medium
- 2.3. Sterilization techniques
- 2.4. Culture conditions
- 2.5. Culture routine
- 2.6. Determination of some growth characteristics
of plants
- 2.7. Anatomical investigations and scanning electron
microscopy (SEM)
- 2.8. Statistical analysis

2.1. PLANT MATERIAL

2.1.1. Seedling-plant material

(a) *Sources*: *Pinus brutia* Tenore, the Calabrian pine.

Seeds were collected from 'Plus' trees no. 45 and 34 at the Zawita Forest, in Mosul province in the northern part of Iraq, at an altitude of 1000 m.

Seeds, with mean dimensions (width and length) of 5.5 and 8.5 mm respectively, were germinated, after stratification in the refrigerator for two weeks, and soaking for 24 h in tap water. The seeds were sown in a mixture of 1:1 sand and peat (granulated sphagnum moss peat, pH 4.0), covered with a thin layer of the soil mixture and placed in a greenhouse (18 h-photoperiod, $20\pm 5^{\circ}\text{C}$ by day and $16\pm 3^{\circ}\text{C}$ at night, temp.) in the Department of Forestry and Natural Resources.

(b) *Excision of explants*

Depending on the study, explants were excised from sterilized seedlings (see sterilization techniques, 2.3.2) of different age classes.

(i) *Excision of needles from seedlings*

Needles (8-30 mm), from sterilized seedlings (up to 12 months old), were each gently pulled off from the upper region of the shoot, and cultured on different media, with various amounts of cytokinin.

(ii) *Excision of pieces from seedling-shoots*

Shoot tips 20-25 mm long from sterilized seedlings (10 weeks old), were excised, and carefully isolated from the surrounding needles. These were then cultured either as: A, shoot pieces without their apical buds, or B, entire shoot pieces including apical buds. In another study, explants from 6 months old seedlings were also used.

2.1.2. Mature plant material

(a) *Sources*: *Pinus brutia* Tenore, the Calabrian pine.

Seeds were collected from 'plus' tree no. 7 at Troodos, Adelphi Forest, Laghoudera in Cyprus, at an altitude of 1500 m. The seeds were stratified, soaked, and germinated as described above. They were sown on a mixture of 1:3 sand:peat in a greenhouse (16 h-photoperiod, 15°C by day and 12°C at night, min. temp.) at the Royal Botanical Gardens (RBG), Edinburgh. The seedlings, which had been grown in pots (containing 1:3:2 parts of sand:peat:sterilized loam) for 2 years under the same greenhouse conditions, were planted out in the nursery at the RBG.

(b) *Collection of explants*

In Edinburgh, bud break occurs in May or June. Lateral branches (10-20 cm length), bearing the newly-formed shoots and fascicle needles (20 mm length), were collected from 10 years old trees in June. In another study, material was collected later in July, so that the fascicle needles were more developed (40 mm length). The lateral branches were brought to the laboratory in plastic bags where they were stored at 4°C for one week before culture.

(c) *Excision of explants*

The fascicle needles, after sterilization (see sterilization techniques, 2.3.3), were carefully excised intact from the shoots, and to minimize contamination, the ensheathing scale leaves were removed.

2.2. NUTRIENT MEDIUM

2.2.1. Choice and modifications to the nutrient medium

It is important to choose a medium on which the species can be cultured successfully. Furthermore, the composition of most media can either support the maximum growth rate or induce organogenesis, but rarely both. Results of earlier work on the nutrient requirements, needed to achieve a high morphogenetic response with cultured embryos and cotyledons of *Pinus brutia* Ten., have indicated the superiority of a modified Schenk and Hildebrandt (SH) medium over the other media tested (Abdullah, Yeoman and Grace, 1985). Yellow shoots are occasionally produced on this medium. However, an increase in the iron content of SH medium to the level used in Murashige and Skoog medium (Murashige and Skoog, 1962) has proved to be effective in overcoming this problem (Abdullah et al., 1985). The composition of the medium finally adopted is listed in Table 2.2.1.

2.2.2. Preparation of stock solutions for the nutrient medium

The chemicals listed in Table 2.2.1 were obtained from BDH Ltd. (except for the vitamins from Sigma Ltd., and Bacto agar from Difco). The stock solutions for the macro elements, iron, and vitamins were made up at 20X, and the micro elements at 100X, of the final concentration required. Each stock solution was made up using 750 ml distilled water in a 1 litre volumetric flask, and each chemical was added and dissolved before the next one was added to avoid precipitation. The stock iron solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 500 ml of distilled water, and the NaEDTA was dissolved in a separate flask in 500 ml of distilled water. Once dissolved the two were added



Table 2.2.1. Composition of Schenk and Hildebrandt medium modified to meet the nutritional requirements of cultured embryos and cotyledons of *Pinus brutia* Ten. (Abdullah et al., 1985).

Constituents	mg l ⁻¹
Macro elements	
KNO ₃	2500.0
MgSO ₄ .7H ₂ O	400.0
NH ₄ H ₂ PO ₄	300.0
CaCl ₂ .2H ₂ O	200.0
Micro elements	
MnSO ₄ .4H ₂ O	20.0
H ₃ BO ₃	5.0
ZnSO ₄ .7H ₂ O	1.0
KI	1.0
CuSO ₄ .5H ₂ O	0.2
NaMoO ₄ .2H ₂ O	0.2
CoCl ₂ .6H ₂ O	0.2
Iron	
FeSO ₄ .7H ₂ O	27.8
Na ₂ EDTA	37.3
Organic constituents	
Thiamine HCL	5.0
Nicotinic acid	5.0
Pyridoxine HCL	0.5
Myo-inositol	1000.0
Sucrose	30000.0
Agar	6000.0

together to make up 1 litre. All other stock solutions were made up to 1 litre. Subsequently, 50 ml aliquots, for each stock solution, were frozen in separate specimen containers (50 ml volume).

2.2.3. Preparation of stock solutions for growth substances

The growth substances used in these studies were either cytokinins as: 6-benzylaminopurine (BAP), Kinetin (Kin), and 2-isopentenylpurine (2iP); or auxins as: 1-naphthaleneacetic acid (NAA), and Indole-3-but^yric acid (IBA) (all obtained from Sigma Ltd.). Each solution was made up as a stock solution at a concentration of 100 mg l⁻¹, and dilution was made when the medium was prepared. Each compound was first dissolved in a small amount (2 ml) of either 1.0 M NaOH (for IBA, BAP, and 2iP), 1.0 M KOH (for Kin), or 100 % ethanol (for NAA). The small amount of solvent containing the dissolved growth substance was carefully added drop by drop to a beaker of stirred distilled water, and each of the stock solutions was then made up to 1 litre in a volumetric flask. Finally, it was divided into 50 ml aliquots, and each was stored at 4°C. All stock solutions were replaced every 3 weeks.

2.2.4. Preparation of other additives

Various additives were used in these studies to overcome browning or to stimulate organogenesis in cultures of mature fascicle needles. Occasionally, activated charcoal was used to stimulate shoot formation from induced adventitious and axillary buds on explants of this species. The additives employed in these studies were as follows :-

1. Hydrogen peroxide (H₂O₂, 100 Vol.)

Sigma Ltd.

2.L-Ascorbic acid 'AnalaR' (ASA)	BDH Ltd.
3.Soluble Polyvinylpyrrolidone (PVP-40 mol. wt. 40,000)	Sigma Ltd.
4.Insoluble Polyvinylpolypyrrolidone (PVP high mol. wt.)	Sigma Ltd.
5.Indole-3-butric acid (IBA)	Sigma Ltd.
6.3-methyl-1-phenyl-5-pyrazolone (PPZ)	BDH Ltd.
7.Malonic acid (propanedioic acid) (MCA)	Sigma Ltd.
8.Sodium diethyldithiocarbamate (SDD)	Sigma Ltd.
9.Activated charcoal (decolorizing powder) (AC)	BDH Ltd.

When included in the nutrient medium, they were weighed out, and dissolved with the other components when the final medium was prepared. When used as soaking solutions, they were dissolved in distilled water at various concentrations. For each soaking treatment, 25 ml of the solution was added to a 100 ml flask covered with aluminium foil, and autoclaved.

2.2.5. Preparation of the nutrient medium and cytokinin solutions

To make up 1 litre of culture medium, the stock solutions were first defrosted (by placing the specimen containers in cold water and then increasing the temperature of the water), and each component (50 ml lots of macro, iron, vitamins, and 10 ml of the micro stock solution) was added successively to 700 ml of distilled water in a 1 or 2 litre flask. Next, the sucrose was added (at levels of either 0.8, 2, or 3%), and the growth substances were then added (a dilution of 10 ml of growth substance stock solution in 1 litre of medium gives a concentration of 1 mg l^{-1}). Whenever other additives were required in the medium, these were added before the solution was made up to final volume. The pH of the solution was adjusted to 5.6 - 5.8 (using either

0.1 M of NaOH or HCL). The solution was either autoclaved directly (for liquid medium), or after Bacto agar of 6 g (0.6 %) had been added to solidify the medium. Media to be poured into sterile Petri dishes after autoclaving were usually autoclaved in large containers (500 ml medium in 1 litre flask) covered with aluminium foil. Otherwise, the nutrient medium was directly poured into the containers (250 ml flasks, 24 x 150 mm test tubes) with agar and autoclaved.

Cytokinin at high concentrations for pulse treatments was added to stirred distilled water and each concentration was made up to 250 ml in a 500 ml flask so as to obtain cytokinin solutions at concentrations of 50, 100, 200 and 400 mg l⁻¹. Each solution was divided into 25 ml aliquots in 100-ml flasks and autoclaved after the pH was adjusted to 4.5 - 5.0.

2.3. STERILIZATION TECHNIQUES

Tissue or organ culture techniques must be performed under entirely aseptic conditions. This requires the sterilization of all instruments, glassware, nutrient media, and culture bench before use. The tissue to be cultured under sterile conditions must be free of micro-organisms. However, methods needed to remove all these micro-organisms should not damage the plant system. In order to achieve the above objectives the following techniques were followed.

2.3.1. Sterilization by heat

Nutrient media, applied growth substances (none of them are heat-labile), soaking solutions, instruments (forceps, tweezers, scalpels), Whatman filter paper, distilled water and all the containers used in other sterilization techniques (2.3.2 and 2.3.3) were autoclaved at 121°C for 20 min. at a steam pressure of 15 Psi. The autoclaved filter paper was then dried in an oven at 60°C for 1-2 h.

2.3.2. Sterilization with hypochlorite-peroxide

The greenhouse-grown seedlings (up to 12 months old) were surface sterilized through two stages. Shoots were cut off, washed thoroughly in tap water, and the cut ends sealed with wax (Fisons). The sealed shoots were surface sterilized by immersion for 7 min. in a 7 % (v/v) solution of sodium hypochlorite (BDH Ltd.), re-sterilized in 6 % 100 vol. hydrogen peroxide for 4 min., and rinsed four times in sterile distilled water. Needles and shoot pieces which were excised were dried on sterile filter paper before culture.

2.3.3. Sterilization with ethanol-mercuric chloride

The technique described above (2.3.2) was not effective in completely removing contaminants on cultures of field-grown plant material. However, a preliminary study indicated the effectiveness of a treatment using ethanol and mercuric chloride (Table 2.3.1).

Newly-formed shoots (2-4 cm length) were cut from the lateral branches, washed with running tap water (3-4 min.), and the cut ends sealed with wax. After a rapid presterilization in 70 % (v/v) ethanol in distilled water for 2 min., the explants were then treated for 10-15 min. in 0.3 % solution (v/v) mercuric chloride (BDH Ltd.), and finally rinsed 4-5 times in sterile distilled water. Fascicle needles were removed from the shoots, and ensheathed scale leaves were carefully removed from the fascicle base so as to minimize the possibility of contamination. Explants were cultured after they had been dried on autoclaved filter paper.

2.3.4. Sterilization with ethanol

Ethanol (70 %) was used to wipe the working surface and the sides of the work-bench before and after the use, and to sterilize disposable gloves usually worn during the manipulation of cultures. All instruments, when not in use, were kept in a jar containing 100 % ethanol. These were flamed immediately before use so as to remove the ethanol and remove any contaminants.

2.3.5. Sterilization by ultra-violet light

All culture manipulations were performed either in a laminar flow cabinet (design as Flow Labs. Irvine, Scotland) or in a 'sterile'

Table 2.3.1. Effects of various sterilization procedures on the removal of contaminants from excised tree-fascicle needles of *Pinus brutia* cultured for 10 weeks on an initiation medium. 200 explants were used for each treatment.

Procedures ***	% of contamination
8% NaOCl* (15 min.)-- 70% ETOH* (2 min.)	80
10% NaOCl* (10 min.)-- 70% ETOH* (2 min.)	100
10% NaOCl* (10 min.)-- 5% H ₂ O ₂ * (15 min.)	90
8% Ca(OCl) ₂ ** (15 min.)-- 70% ETOH* (2 min.)	60
8% Ca(OCl) ₂ ** (10 min.)-- 5% H ₂ O ₂ * (15 min.)	100
95% ETOH* (30 Sec.)	80
0.1% AgNO ₃ ** (3 min.)-- 0.1% NaCl** (3 min.)	100
70% ETOH* (2 min.)-- 8% NaOCl* (15 min.)	85
70% ETOH* (2 min.)-- 8% Ca(OCl) ₂ ** (15 min.)	85
70% ETOH* (2 min.)-- 0.05% HgCl ₂ ** (15 min.)	40
70% ETOH* (2 min.)-- 0.15% HgCl ₂ ** (15 min.)	30
70% ETOH* (2 min.)-- 0.30% HgCl ₂ ** (10 min.)	15

* Z= (v/v) solution with water, ** Z= (w/v) solution with water.

NaOCl= sodium hypochlorite, Ca(OCl)₂= calcium hypochlorite,

AgNO₃= silver nitrate, NaCl= sodium chloride, ETOH= ethanol,

H₂O₂= hydrogen peroxide, HgCl₂= mercuric chloride.

*** Explants, after each sterilization, were rinsed 4 times in sterile distilled water.

culture room reserved for such purposes. This room was irradiated with two ultra-violet lights (producing light of a wavelength of approximately 300 nm) for a minimum period of 20 min. before entry and was kept under a continuous positive pressure by an inflow of sterile air to prevent any inward drift of air-borne microbes.

2.4. CULTURE CONDITIONS

The culture conditions, including strength of the nutrient medium, sucrose levels, culture containers, and environmental conditions, were all adjusted according to the requirements of cultures at each stage of plant regeneration.

2.4.1. Bud initiation

(a) Culture medium and containers

During initial culture and subsequent subculture, explants were either incubated in the modified SH liquid-medium (25 ml liquid medium in 100 ml flasks (Fisons), slowly agitated at 30-35 r.p.m. on a gyrarotory shaker), or grown on a solidified medium with 6 % Bacto agar (10 or 20 ml agarified medium in 50 x 20 or 90 x 15 mm Sterilin Petri dishes respectively). In all these studies, media were applied at full-strength (3 % sucrose) supplemented with growth substances, and a batch of 10 needles or 3 shoot pieces were cultured in each container. The plates were sealed with Parafilm[®] "M" (Aldrich Chemical Co. Inc.), and maintained in a controlled environmental chamber.

(b) Environmental conditions

The adjustment of culture temperature was made taking into consideration the temperatures measured inside the containers (Petri dishes, flasks), as differences of 4°C have already been found between the temperature of the growth chamber and that inside the plates (Abdullah et al., 1985). At this stage of plant regeneration, the cultures were incubated at an average temperature of $23 \pm 1^\circ\text{C}$ ($26 \pm 1^\circ\text{C}$ inside the plates).

Investigation of the photoperiodic requirement of the cultured

embryonic tissues of *Pinus brutia* has clearly demonstrated that a photoperiod of 16 h is more favourable in eliciting a high yield of adventitious buds than is continuous illumination (Abdullah et al., 1985). Therefore, this photoperiod was employed with all explant sources used for the culture of this species. Explants of *Pinus brutia* used for the initial stage of regeneration were incubated under a photon flux density of $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ (400-700 nm). The light source consisted of a bank of 6 white fluorescent tubes (85 W, Osram), interspersed with 3 (60 W, Philips) incandescent lamps.

2.4.2. Bud elongation and shoot formation

(a) Nutrient medium and containers

Induced buds which required further elongation to complete shoot formation, were subcultured into 250 ml flasks (Fisons) containing 50 ml of a half-strength medium supplied with 2 % sucrose (0.6% agar), but without growth substances.

(b) Environmental conditions

The light regime (photoperiod and intensity), and the temperature employed for this stage were the same as that for the initiation stage.

2.4.3. Rooting treatment and root induction

(a) Nutrient medium and containers

For the rooting treatment, induced shoots were incubated in 150 x 24 mm test tubes (Fisons) containing 30 ml half-strength medium supplied with 2 % sucrose (0.6% agar), after it had been supplemented with different combinations of growth substances. For root induction, the treated shoots (each 5 shoots) transferred into 250 ml flasks

containing 100 ml of the same medium, but devoid of growth substances, and including 0.8% sucrose (0.6% agar).

Alternatively, for rooting under non-sterile conditions, a group of the treated shoots was subcultured into 250 ml flasks containing tap water (under non-sterile conditions), in which each of the 3 shoots was supported on a filter-paper bridge on the top of each flask with the shoot base in contact with the surface of the water. Another group of treated shoots were transferred into plastic trays containing tap water aerated by a small electric pump (Hy-Flow air pump). The shoots were suspended on polystyrene foam bridges with the shoot base in contact with the surface of the aerated tap water, which was changed at 2-week intervals.

(b) Environmental Conditions

For root treatments, the shoots were maintained in a growth chamber with the same light and temperature conditions as that applied in the initiation stage. However, for root induction, the treated shoots were maintained in a controlled environmental chamber at a temperature of $21 \pm 1^{\circ}\text{C}$ under a 16 h photoperiod (fluorescent light only) with a photon flux density of $70 \mu\text{mol m}^{-2} \text{s}^{-1}$. To maintain a high humidity around the cultures, for rooting under non-sterile conditions, containers were covered by a plastic sheet.

2.4.4. Hardening off and establishment of plantlets

(a) Nutrient medium and containers

Rooted shoots, after 8-10 weeks on root-induction media (for the final 1-2 weeks at a photon flux density of $95 \mu\text{mol m}^{-2} \text{s}^{-1}$), were potted in 89 mm pots. The medium used was 1:1 sand and peat

(granulated sphagnum moss peat, pH 4.0). Plants were hardened off under a mist irrigation system for 1 month. The plantlets were then transferred to the greenhouse for another 1 month, where they were irrigated as usual. Eventually, they were repotted in 130 mm pots with the above soil mixture, and maintained for another 4 months.

(b) Environmental Conditions

Rooted shoots, for hardening off, were maintained in a mist chamber illuminated at a 18 h photoperiod with a mercury fluorescent lamp (400 W, MBFR/U, Thorn) at a temperature of $25 \pm 2^{\circ}\text{C}$ by day and $20 \pm 2^{\circ}\text{C}$ at night. The greenhouse was also illuminated by similar lamps. The greenhouse temperature was about $20 \pm 5^{\circ}\text{C}$ by day and $16 \pm 3^{\circ}\text{C}$ at night.

2.5. CULTURE ROUTINE

2.5.1. Adventitious shoot formation on cultures of seedling-needles

(a) Initiation of adventitious buds

a.1. Cytokinin treatments

Investigations were carried out to explore the morphogenetic responses of seedling-needles *in vitro* to the application of cytokinins.

i. Application of different types and concentrations of cytokinin

The production of adventitious buds on excised needles (from 8 weeks old seedlings) was evaluated by incorporation in the agarified medium of 0, 1, 5, and 10 mg l⁻¹ each of BAP, Kin, and 2iP.

ii. Mixtures of cytokinins

Mixtures were applied by incorporating in the agarified medium 0.5 + 0.5 and 1.0 + 1.0 mg l⁻¹ of a mixture of BAP + Kin, BAP + 2iP, and Kin+ 2iP.

iii. Inclusion of auxin with applied cytokinins

All the treatments in (i and ii) were tested with or without 0.01 mg l⁻¹ NAA.

iv. Seven treatments (in mg l⁻¹, BAP (1), BAP (5), BAP (10), Kin(10), 2iP (10), BAP + Kin (0.5 + 0.5), and BAP + Kin (1.0 + 1.0), selected from both procedures, were retested on agarified medium using the same source of explants.

v. Pulse treatments

In another series of experiments, the morphogenetic responses of cultured needles (excised from 8 weeks old seedlings) were investigated to the influence of BAP at high concentrations (50, 100, 200, and 400

mg l⁻¹) pulses to the applied explants with continuous shaking for 2 h. A time-course experiment with the best concentration of BAP was then carried out over a pulse-time of 1, 2 and 4 h.

In all these studies, explants were either cultured directly, and subcultured at 4 week intervals on CK-containing medium, or first pulse treated and rinsed in sterile distilled water before culture, and subcultured at 4 week intervals on CK-free medium.

a.2. Age of donor seedlings

To examine the relationship between the budding potential of seedling needles and their degree of differentiation (age of donor seedlings), a factorial study was performed on the influence of BAP, supplied at concentration of 5 and 10 mg l⁻¹, on the morphogenetic potential of excised needles from seedlings of different age classes (1, 3, 6, and 12 months old seedlings).

a.3. Physical state of applied medium

The same treatments listed in (iv) were re-examined in the liquid medium as described before (culture conditions, 2.4.1, (a)).

a.4. Final account using optimal procedure

A final account for the yield of adventitious buds on 1440 needles (excised from 8 weeks old seedlings) derived from the two 'plus' trees (No.45, and 34) was made using the most favourable medium (agarified medium) and cytokinin treatment (BAP at level of 10 mg l⁻¹, and BAP + Kin at level of 1 mg l⁻¹ for each), determined as described above. The production of adventitious buds on these treatments was compared with

that obtained on treatment time and concentration of BAP chosen as the best pulse induction treatment.

(b) Bud development and shoot formation

For bud development and shoot formation, induced buds from various sources were used. Some had been cultured for 8 weeks on CK-containing medium, others had been cultured for 8 weeks on agarified CK-free medium for the pulse treatment. Others were cultured for 3 weeks in liquid medium followed by 8 weeks on agarified CK-free medium. Irrespective of source, they were transferred to media with the following composition:

Medium A= half-strength medium + 2 % sucrose

Medium B= half-strength medium + 2 % sucrose + 1 % activated charcoal

Shoot elongation was evaluated after 4 weeks on the latter media. 250 buds were used for each treatment.

Evaluation was made of the percentage of cultured needles forming adventitious buds; percentage in which excess callus growth was induced, and number of induced buds per 100 cultured needles. This evaluation was made after the total induction period mentioned above for each study.

(c) Bud multiplication

For bud multiplication, the induced buds were recultured on CK-containing medium (BAP of 10 mg l^{-1} , or BAP + Kin each at level of 1 mg l^{-1}) for another 4-6 weeks, where a final account of the number of induced buds per 100 cultured needles was made.

2.5.2. Axillary shoot formation on seedling-shoot explants

(a) Axillary bud and shoot development

To induce the growth of primary axillary buds, which were already present along the shoot axes of seedlings, and to promote the subsequent induction of secondary buds on seedling-shoot pieces that initially had been incubated on agarified medium, the following treatments were applied :-

a.1. Application of Cytokinins

Cytokinin (BAP) was included in the induction-medium, as an exogenous stimulus for axillary bud production and development, either singly at a concentration of 3 or 5 mg l⁻¹, or together with kinetin (Kin) each at level of 1 mg l⁻¹.

a.2. Surgical removal of apical buds

To examine the possibility of increasing the production of axillary buds through mechanical removal of the apical bud so as to overcome the dominance of the latter over axillary buds, explants were cultured with or without their apical buds.

The shoots which developed from primary axillary buds on cultured explants and secondary buds induced at axils of each of the new primary shoots were evaluated (both the number and the length) after a total incubation time of 6 weeks.

(b) Shoot formation from secondary buds

Following the latter evaluation, the secondary buds were subcultured into media described for shoot formation on cultured needles (culture routine, 2.5.1, b).

(c) Shoot multiplication

Two techniques were used for shoot multiplication. Firstly, secondary buds were transferred every 4 weeks onto a medium without cytokinin, and after 12 weeks a final account of the number and length of buds was made. Secondly, individual primary shoots, which had already produced a crop of secondary shoots, were maintained on a medium with BAP + Kin (1 mg l^{-1} for each) for 4-6 weeks. To encourage elongation, newly produced buds at axils of the primary shoots, were subcultured for 2 weeks onto SH medium supplied with 1 % activated charcoal.

2.5.3. Formation of shoot-buds on mature fascicle needles

(a) Initiation of shoot-buds

a.1. Prevention of browning of explants

Explants frequently turned brown after isolation and culture, and then died. To prevent browning of cultured explants several approaches were followed:

(i) Soaking of explants before culture

Explants, each 20 fascicle needles, after sterilization and excision, were soaked in flasks containing one of several soaking solutions, with continuous shaking as shown below:-

<i>Soaking solutions</i>	<i>Soaking time</i>	<i>Purpose</i>
1. 2% Hydrogen peroxide	1 h	Removal of phenolic substances
2. 0.005% Ascorbic acid	1 h	Anti-oxidant to modify redox potential
3. 0.5 or 1 % Insoluble PVP	1 h	Absorption of phenolic substances

4. 0.5 or 1% soluble PVP-40 1 h Absorption of phenolic substances
5. Distilled water 1,2,4,8, Leaching of phenolic substances
12, or 24 h
6. 5 or 100 mg l⁻¹ 24 h or Stimulation of organogenesis
solution of IBA 15 min.
7. 10 or 100 mg l⁻¹ 24 h or Stimulation of organogenesis
solution of PPZ 15 min.
8. 10 or 100 mg l⁻¹ 24 h or Stimulation of organogenesis
solution of malonic acid 15 min.
9. Explants were cultured without soaking treatment (Control).

(ii) Application of anti-browning substances to culture media

The soaked explants were then cultured on full-strength medium supplemented with a mixture of BAP + Kin (each at level of 5 mg l⁻¹).

Otherwise, explants were directly cultured on the latter medium after adding either a chelate (SDD) or polyamid (PVP, soluble and insoluble) at various concentrations for an incubation time of 10 weeks.

1. 0.5 - 1 % soluble PVP-40
2. 0.5 - 1 % insoluble PVP
3. 200 mg l⁻¹ of SDD (chelating agent to inhibit the action of
polyphenol oxidase enzymes).

Explants were subcultured at 3 week intervals onto fresh media. A final account of frequency of brown needles was made after a total incubation time of 10 weeks.

*(iii) Application of anti-browning substances to cultures of
seedling-needles*

It was considered likely that such substances (applied in *i* and *ii*) might inhibit development, and so the influence of the best treatments prevented browning (selected from the above experiments) on the morphogenetic response of seedling-needles (8 weeks old) was also considered. Accordingly, the following treatments were retested:-

1. 0.5% insoluble pvp (included with the culture medium).
2. Soaking treatment in 5 mg l^{-1} of IBA for 24 h before culture.
3. Soaking treatment in 10 mg l^{-1} of PPZ for 24 h before culture.
4. 200 mg l^{-1} SDD (included with the culture medium).
5. Control.

Explants were either soaked or cultured directly on the same medium as in (*ii*).

(iv) Application of different levels of sodium diethyldithiocarbamate to cultures of seedling-needles

The morphogenetic response of seedling-needles (8 weeks old) cultured on the same CK-medium as that applied in (*ii*) was again examined with regard to the inclusion of SDD at concentrations of 50, 100, 150, 200, 250 and 300 mg l^{-1} , to find the optimum concentration maintaining both a high frequency of organogenesis and a low level of browning.

Explants, in both experiments (*iii*) and (*iv*) were subcultured at 4 week intervals onto fresh media for a total incubation time of 10 weeks, where a final account for the morphogenetic responses on cultured needles was made.

(v) *Application of sequence-treatments to cultures of mature fascicle needles*

Once the best type and concentration of anti-browning substance was determined, the possibility of improving this procedure was investigated. This involved the incubation of the fascicle needles on media with different additives each for various times of incubation as follows :-

1. Medium supplied only with cytokinins (BAP + Kin each at level of 5 mg l^{-1}) for 10 weeks.
2. The same as in (1) with inclusion of 0.5% insoluble PVP for an incubation time of 10 weeks.
3. The same as in (1) with inclusion of 150 mg l^{-1} of SDD for an incubation time of 10 weeks.
4. The same as in (3) for an incubation time of 6 weeks, but the SDD was replaced by 0.1 % of insoluble PVP for an incubation time of 4 weeks.
5. The same as in (2) for 8 weeks, after pretreatment of explants on a medium devoid of cytokinins, but containing 1% activated charcoal.
6. The same as in (3) for 8 weeks, after pretreatment of explants as in (5).

An explants were subcultured at 3-week intervals onto fresh media. Finally, the percentage of browned explants, and the morphogenetic responses of cultured explants were followed at incubation times of 6 and 10 weeks.

a.2. Effect of season of collection

Explants collected at different times (see plant material, 2.1.2, (b)) were maintained on a medium containing cytokinins (as in v), with 150 mg l^{-1} of SDD for a total incubation of 10 weeks. Morphogenetic responses and frequency of browning of cultured needles were evaluated.

(b) Further shoot elongation and multiplication

Induced shoot-buds, for shoot elongation, were cultured on half-strength modified SH medium with or without BAP+Kin (1 mg l^{-1} for each). elongated shoots were subsequently evaluated for their height after total incubation times of 4 and 10 weeks. However, for shoot multiplication, elongated shoots with mean height of either 10-15 or 20-28 mm, were incubated individually on a medium supplemented with BAP+Kin (2.5 mg l^{-1} for each) for 8 weeks where a final account for number of multiplied buds and shoots per explants was made. 30 and 45 explants were used for each treatment in the above studies respectively.

2.5.4. Regeneration of plantlets

I. Adventitious root formation on axillary-derived shoots

The axillary-derived shoots represent a more homogeneous population than adventitious-derived shoots, and were used to explore the action of exogenous auxins and cytokinin on the initiation and development of adventitious roots and on subsequent plant development and performance.

(a) Application of growth substances

a.1. Application of various types and concentrations of auxins

Axillary-derived shoots (2 cm in height) were cut diagonally to expose the base of the stem. They were then subjected to media containing various combinations of two auxins as follows :-

NAA at concentration of 0.0, 0.25, 0.50, and 1.0 mg l⁻¹

IBA at concentration of 0.0, 1.0, and 2.0 mg l⁻¹

a.2. Application of different concentrations of cytokinin

The inclusion of exogenous cytokinin at low levels with applied auxins was also tested. BAP was added to the above-mentioned combinations of auxins at the following concentrations: 0.0, 0.05, and 0.5 mg l⁻¹.

After shoots had been treated as above for 12 days, they were subcultured onto media devoid of growth substances for a total incubation time of 8 weeks, where the frequency of rooting, and root number per plant were evaluated.

For 6 months or more after transplanting plantlets from the sterile agar-medium into soil, the characteristics of induced roots and shoots were recorded as follows:-

(1) Branching of induced roots and mycorrhizal root formation

The exhibition of dichotomous branching by the induced roots was used in this study as a conspicuous diagnostic feature of infection and establishment of the mycorrhizal habit in pine species (Harley and Smith, 1983).

(i) The frequency of branched and mycorrhizal plantlets was evaluated after the hardening-off period (1 month) of the rooted shoots.

(ii) This was again recorded after another month after transplanting to greenhouse conditions.

(iii) A final record of the percentage of plantlets that were mycorrhizal was made after another 4 months, and the the degree of such expression was scored as follows:-

1. Root system with out branches.
2. Root system with few branches.
3. Root system with good dichotomous branching.
4. Root system with very good dichotomous branching.

(2) Other characteristics of the induced roots

A final evaluation was made after the 6 month period as follows:-

- (i) Number of induced roots per root system.
- (ii) Root elongation rate per month (cm/month).
- (iii) Rate of shoot height growth (cm/month).
- (iv) Relationship between shoot height growth rate and rank score of mycorrhizal formation on the induced roots
- (v) Survival percentage of the established plantlets.

(b) Application of different root-induction media

An alternative procedure of rooting the shoots under non-sterile conditions was tried using the cheapest possible substrate (tap water). Axillary derived-shoots (2 cm in height), after being treated for 12 days on a medium supplied with a combination of NAA + IBA + BAP each at concentration of 1 + 2 + 0.05 mg l⁻¹ respectively, were subcultured onto the following root-induction media:-

1. Sterile agar-medium (control, as described in culture condition, 2.4.3).

2. Tap water
3. Aerated tap water.

The rooting of the treated shoots incubated on the above media, and the subsequent performance of the plantlets were evaluated as follows:-

(i) Quantitative and qualitative characteristics of induced roots

The rooting frequency of the treated shoots was followed at weekly intervals for a total incubation time of 10 weeks. A final evaluation was also made of the length and number of induced roots.

(ii) Callus growth

The percentage of treated shoots that proliferated excess callus growth was recorded after 10 weeks incubation.

(iii) Survival of regenerated plantlets

Survival of regenerated plantlets was also assessed 6 months after transplanting the plantlets from each of above media into soil in the greenhouse.

**II. Adventitious root formation on adventitious-derived shoots
grown from seedling-needles**

The induced shoots, for rooting, were treated for 12 days on a combination of NAA + IBA + BAP of 1 + 2 + 0.05 mg l⁻¹ respectively. All other aspects of rooting treatments; root induction, hardening off, and establishment of plantlets were just as described before.

**III. Adventitious root formation on shoot induced on mature
fascicle needles**

The same above sequence of methods for rooting and plantlet

establishment was followed except that rooting treatment was applied for 12 and 18 days.

2.5.5. Patterns of growth and development of plantlets

A final investigation was made to assess the possible differences in the pattern of development of regenerated plantlets compared to that of seedlings. Various aspects of growth and development after 12 months in soil, were evaluated as follows :-

1. Axillary plantlets

Axillary-derived shoots (2 cm in height) which were produced via meristematic tissues on shoot pieces excised from seedlings grown from seeds of 'plus' tree no. 45, were treated for 12 days on a medium supplied with a combination of NAA + BAP at concentration of 1.0 + 0.05 mg l⁻¹ respectively. The treated shoots were then rooted on a sterile agar medium as described earlier in this chapter.

2. Adventive plantlets

Adventitious buds were produced on embryonic tissues. Non-meristematic tissue explants were dissected from seeds of 'plus' tree No. 45), elongated into normal shoots (2 cm in height) (for details see Abdullah et al., 1985), and rooted as described above.

The rooted shoots, of both origins, were potted into 89 mm pots, and hardened off on a mist bench (as described in culture conditions, 2.4.4).

3. Seedlings

At the same time, stratified seeds from the same 'plus' tree (No. 45) were sown in pots (89 mm) on the mist bench.

After 1 month on the mist bench, both the plantlets and germinated

seedlings were transferred into the greenhouse. All these plants, after another 3 months, were repotted in 130 mm pots and maintained for another 8 months in the greenhouse. A final evaluation of the shoot and root characteristics of the three groups of plants was made as follows:-

- i. Shoot height (cm), diameter (mm), and dry weight (g).
- ii. Root length (cm), number, diameter (mm), and dry weight (g)
- iii. Total dry weight of the plants (g).
- iv. Relationship between root dry weight and shoot dry weight for each type of plants (g/g).
- v. Specific leaf area and leaf dry weight ratio ($\text{cm}^2 \text{g}^{-1}$)
- vi. The stomatal resistance of the leaf (s cm^{-1}).
- vii. The time-appearance of fascicle on plants (month).
- viii. Patterns of growth form of plants.

2.6. DETERMINATION OF SOME GROWTH CHARACTERISTICS OF PLANTS

2.6.1. Dry weight of the plants

The shoot, root, or total dry weight of the plantlets or seedlings was obtained by putting the plant material in an oven at 80°C for at least 48 h. They were then weighed, after cooling in a desiccator, placed in the oven for another 24 h, cooled and reweighed. If there was no decrease in weight for any of the plant parts, it was assumed that they were oven dry.

2.6.2. Specific leaf area and leaf dry weight ratio

The leaf area (fascicle needle area) at the end of the experiment was determined using the Leaf Area Meter (Li-3000, LiCOR, Nebraska). To obtain oven dry weights, the leaves were then dried and weighed as described above. To calculate Specific Leaf Area and Leaf Dry Weight Ratio (SLA) the equation given by Hunt (1982) was used.

$$SLA = \frac{L_A}{L_W}$$

where L_A = the leaf area (cm^2).

L_W = the leaf dry weight (g).

2.6.3. Stomatal resistance of leaves

To calculate the stomatal resistance of leaves to water vapour the porometer developed by Beardsell, Jarvis and Davidson (1972) was used. This instrument was suitable for use on the needle-like leaves of conifers. The following equation was used for calculation :

$$r = \left(\frac{100 - 1}{RH} \right) \frac{A}{f}$$

r = stomatal resistance of leaves to water vapour ($s\ cm^{-1}$).

RH = relative humidity in the porometer chamber.

A = the outline area of one surface of the leaves (cm^2).

f = the outflow of air ($cm^3\ s^{-1}$).

2.6.4. Patterns of growth form of plantlets

The growth form of regenerated plantlets and seedlings was classified into four categories as follows :-

- i. Upright growth form.
- ii. Upright growth form, and bushy at the base.
- iii. Plagiotropic growth form.
- iv. Bushy growth form.

2.7 ANATOMICAL INVESTIGATIONS AND SCANNING ELECTRON

MICROSCOPY (SEM)

To find where cell division was initiated (for non-meristematic tissues) and to examine the stages of development of immature buds (for the meristematic tissues), induced explants were prepared at weekly intervals for histological and scanning electron microscopy (SEM) studies. Specimens for anatomical study were fixed in Karnovsky's fixative (Karnovsky, 1965) at 4°C for 24 h for light microscopy (LM), and 36 h for SEM. These received a post-fixation treatment in 1 % (W/V) aqueous osmium tetroxide (Emscope) solution for 2-3 h. Specimens for LM were dehydrated in an ethanol series, followed by propylene oxide (BDH Ltd.), and embedded in Epon (50 ml Epon 812 substitute + 50 ml dodecyl succinic anhydride + 2 ml benzyldimethylamine, Emscope). Sections were cut on a Reichert-Jung Ultracut microtome at a thickness of 0.5 μm . Ultrathin sections were stained with 0.5 % (W/V) toluidine blue (Sigma Ltd.) in 1 % (W/V) Borax (Andrew H. Baird Ltd.) (see Roland, ;1978). Specimens for SEM (Cambridge Stereoscan 250) were dehydrated in an acetone (Fisons) series, and critical point dried under liquid CO₂.

2.8. STATISTICAL ANALYSIS

Data from factorial and non-factorial experiments were subjected to analysis of variance using the GENSTAT package (Lowe's Agricultural Trust, Rothamsted, England). The analysis of data estimated as a percentage or ratio was performed after the logit transformation (Snedecor and Cochran, 1980).

Statistical parameters evaluated included the estimation of standard error and coefficient of variation (CV). Least significant range (LSR) was calculated using Duncan's multiple range test (Duncan, 1955). Correlation studies were made using the PRESTO package (general modelling, graphics and statistics package, R. L. Muetzfeldt, available from the Department of Forestry and Natural Resources). The cultures in all the experiments were set out in a randomized block design. The number of explants used for each individual treatment and each factor investigated ranged from 30-300 and 360-720 respectively.

CHAPTER 3

EXPERIMENTAL RESULTS

3.1 THE PRODUCTION OF BUDS AND SHOOTS

In this part, a series of experiments were carried out to develop methods for initiation, development, and multiplication of adventitious and axillary shoots using explants from seedlings and mature trees. Here, the results are presented within the three sections according to the type of explant used.

Section 3.1.1, comprises investigations into the formation of adventitious shoots which develop on cultured needles which have been excised from greenhouse-grown seedlings. The objective of these experiments was to formulate a protocol of basic cultural conditions for the optimum production of shoots on these juvenile needles. Accordingly, 'screening experiments' were aimed to determine the effects of factors such as type, concentration, and mode of application of exogenous growth substances, as well as testing the nutrient medium in liquid or solid form. The developmental potential of the explants, using optimum culture conditions formulated as described above, is examined in terms of influence of age (physiological state) of the donor seedlings. An analysis of gross anatomical changes and the developmental sequence of adventitious bud formation on the cultured explants is also given. Methods are described which do, in fact, achieve good rates of elongation coupled with high rates of multiplication of the produced buds, and which may form the basis of a practical scheme for further use. An account of the *in vitro* production rate of shoots per single seedling has been made. Once established, these culture conditions were applied for the production of shoots on the other more difficult types of explants.

In both sections 3.1.2 and 3.1.3, attempts are described to re-activate existing dormant meristems on the shoot axes of the

seedlings or those in axils of fascicles explanted from mature trees. In section 3.1.2, a procedure is described for the rapid production of primary axillary shoots and secondary crops of buds and shoots on seedling plant material. The role of exogenous factors, such as applied cytokinins and mechanical abolition of apical dominance of the explants, on increasing the *in vitro* shoot productivity is also examined. Anatomical events associated with the development of these reactivated meristems into 'well-formed' axillary shoots are described. Procedures for shoot elongation and for achieving successive crops of shoots and buds on the induced shoots are also considered.

In section 3.1.3, approaches designed to minimize phenolic browning of the cultured fascicles, whilst maintaining a maximum rate of shoot-bud production are presented. This involved testing various anti-browning agents, their mode of application, and collecting the explants twice during the year. The role of exogenous cytokinins in the elongation and development of induced shoot-buds, and in the multiplication of the elongated shoots, was also examined.

3.1.1 ADVENTITIOUS SHOOT FORMATION ON CULTURED SEEDLING-NEEDLES

As set out in Chapter 1, the vegetative propagation from seedling plant material in conifers enables the regeneration of plants for planting stock started from scarce and costly seeds derived from controlled pollinations, and it may be used where a clonal alternative to reforestation with seedlings is desired (Libby, 1979). In this section, a series of experiments was conducted to investigate the effects of various factors involved in the process of shoot regeneration using seedling-needles of *Pinus brutia*.

A. Initiation of adventitious buds

i. Anatomical observations with the light and scanning electron microscope

Before culture, tissues at the basal region of the needle were apparently parenchymatous (Fig. 3.1.1.1). Using the SEM, a comparison between initially cultured explants (Fig. 3.1.1.2) and those after 1 week of culture on a medium containing cytokinins revealed a marked swelling at the needle base (Fig. 3.1.1.3). From the section it could be seen that cell division had occurred mainly in the layers of parenchyma-mesophyll in the basal area (Fig. 3.1.1.4). Cell division proceeded for longer and became widespread in this region in the following 2 weeks, and formed longitudinal files of meristematic cells (Fig. 3.1.1.5). A bud primordium then began to develop first as an apical growth which formed a protuberance at the needle base, with an associated outgrowth (first needle primordium) that appeared laterally (Fig. 3.1.1.6). Subsequently, other needle primordia grew out from the apical meristem which became elongated (Fig. 3.1.1.7). Also the bud

Fig. 3.1.1.1--3.1.1.12: A developmental sequence of the induction of adventitious buds on excised needles from seedlings of *Pinus brutia* 8 weeks after culture on a modified SH medium supplied with 10 mg l^{-1} BAP. Fig. 1. Longitudinal section of a needle before culture. Note the parenchymatous cells of the basal area (arrows). Fig. 2. An electron micrograph (SEM) of a needle before culture. Fig. 3. Electron micrograph (SEM) of a needle 1 week in culture. Note the swelling of the needle at the base (arrow). Fig. 4. Longitudinal section of a needle 1 week in culture, showing cell divisions (arrows) in the mesophyll layers at the basal area. Fig. 5. Longitudinal section of a needle base 3 weeks in culture. Meristematic zones have been formed (arrows). Fig 6. Needle 4 weeks in culture, see the early development of a bud primordium consisting of apical meristem (AM) associated with one needle primordium (NP).

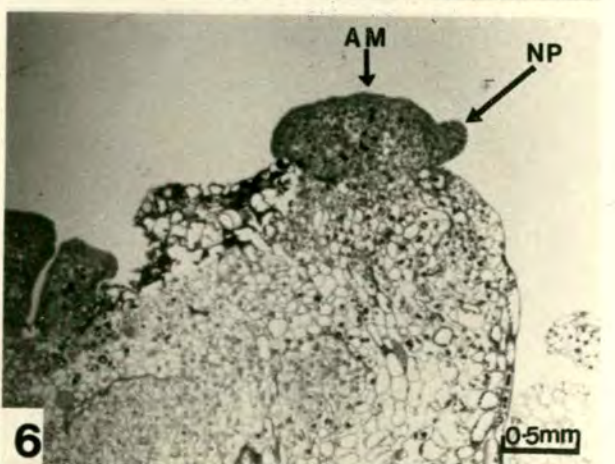
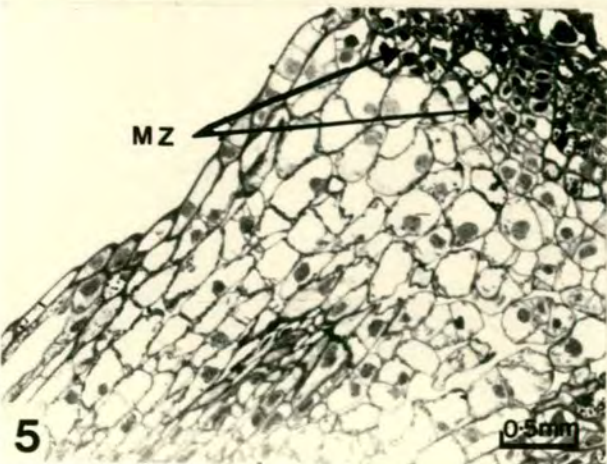
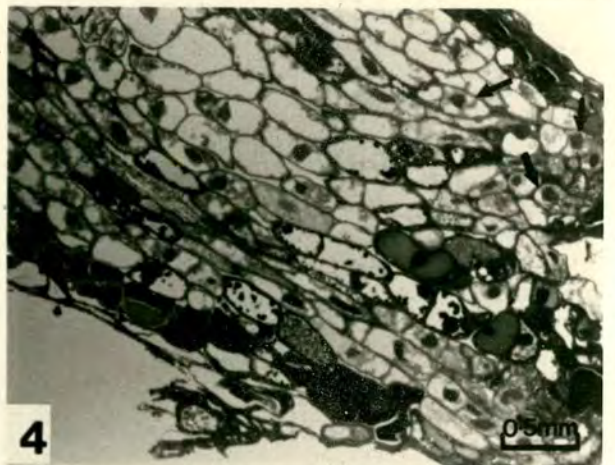
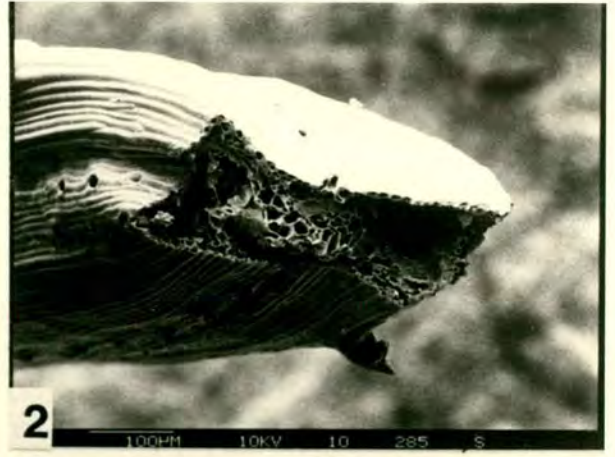
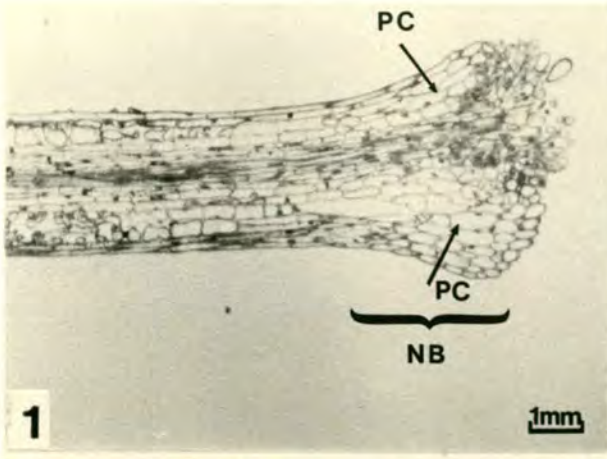


Fig 7. Longitudinal section of a needle 5 weeks in culture showing later stage of development of a bud primordium with apical meristem flanked by two needle primordia. Fig. 8. Early stage of development of a bud primordium into a bud. Note the appearance of the first needle (N). Fig. 9. Electron micrograph for the same stage as in Fig 8. Fig. 10. Electron micrograph and Fig. 11. Longitudinal section of a well-formed bud after 8 weeks of culture. Fig. 12. Multiplication of induced bud on cultured-needles after a total incubation of 14 weeks on BAP-containing medium.



primordium had developed further prior to the appearance of the first needle (Fig. 3.1.1.8 and 3.1.1.9). Under the influence of applied cytokinin (10 mg l^{-1} of BAP) a total incubation time of 8 weeks was required to achieve the induction of well-formed adventitious buds on a cultured needle (Fig. 3.1.1.10 and 3.1.1.11). A further incubation time of up to 14 weeks led to the production of many buds on each cultured needle (Fig. 3.1.1.12).

ii. Influence of growth substances

It was apparent that the cultured needles exhibited different quantitative organogenic responses according to the nature of the growth substance applied.

a. Influence of cytokinins

Both the type and concentration of cytokinin employed in these investigations significantly affected the organogenic potential of the cultured needles. BAP, over a wide range of concentrations, caused a maximal expression of adventive budding on cultured explants. Kin was found to be less potent than BAP, whereas, 2iP was the least effective cytokinin in this respect (Fig. 3.1.1.13). Kin, at a higher level of 10 mg l^{-1} was just about as effective as the BAP at 10 mg l^{-1} , but the cytokinin 2iP, even at its highest level applied (10 mg l^{-1}) was ineffective (Fig. 3.1.1.13). In general, the most effective cytokinin concentration for bud induction, with all cytokinins tested, was 10 mg l^{-1} (Fig. 3.1.1.13). The ability of cultured needles to form buds was also influenced by the interaction between the types and levels of cytokinin applied, and the best result of all was obtained with 10 mg l^{-1} of BAP (Fig. 3.1.1.13).

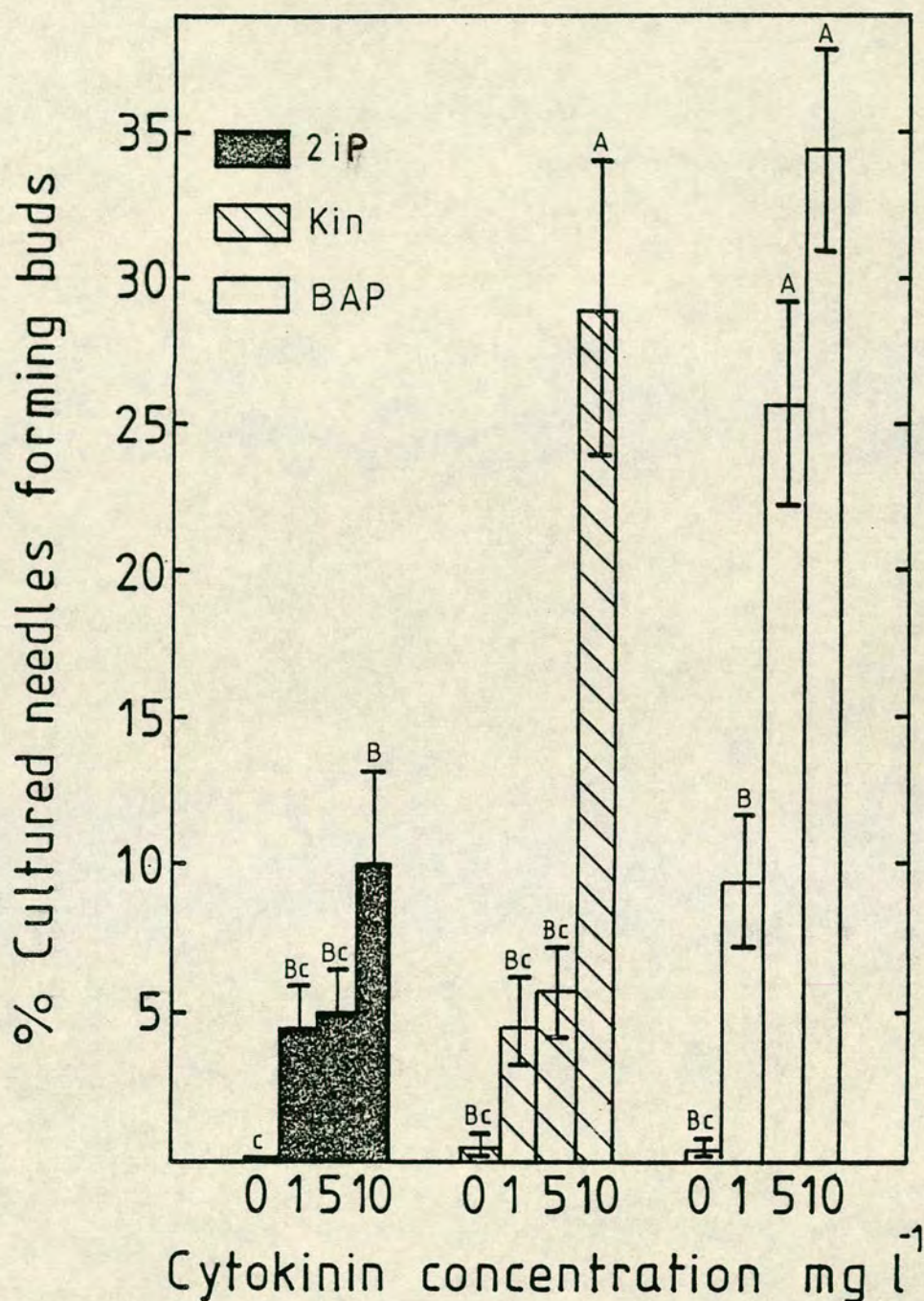


Fig. 3.1.1.13 Interaction of type and quantity of applied cytokinin on the frequency of bud induction on excised needles from seedlings of *Pinus brutia* Ten. Explants were incubated for 10 weeks on cytokinin-containing modified SH medium. 120 explants were used for each treatment. Different letters above any two bars indicate that these two means are statistically different at $P=0.01$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

A mixture of two cytokinins was unable to sustain a higher rate of bud formation than that obtained using separate cytokinins (Fig. 3.1.1.14, 3.1.1.17, and Table 3.1.1.1). However, it was a mixture that evoked the highest number of induced buds on each cultured needle, and mixing BAP with either Kin or 2iP gave a higher production of buds than a mixture of Kin and 2iP (Table 3.1.1.1).

b. Influence of mode of application of cytokinin

A pulse treatment of cytokinin applied to the cultured needles, with BAP at 50 mg l^{-1} , elicited a higher number and rate of production of buds than that obtained on higher concentration of BAP (Table 3.1.1.3). Pulse treatments of 200 and 400 mg l^{-1} BAP promoted excess callus proliferation on the cultured needles, and they were lethal to some explants (Table 3.1.1.3). The morphogenetic responses of pulse treated explants (50 mg l^{-1} BAP) were better with a 2 h pulse-time than with 1 h. No real gain was obtained with a longer pulse time of 4 h (Fig. 3.1.1.16). In all cases, 8 weeks was required for the adventitious buds to complete development.

c. Influence of inclusion of auxin with applied cytokinin

The inclusion of exogenous auxin (NAA), even at the low level applied in this study (0.01 mg l^{-1}), with either a cytokinin alone or a mixture of two cytokinins resulted in a significant increase in callus proliferation rather than the production of adventitious buds on cultured needles (Table 3.1.1.2).

iii. Influence of physical properties of nutrient medium

The morphogenetic yield (both the rate of productivity and number of

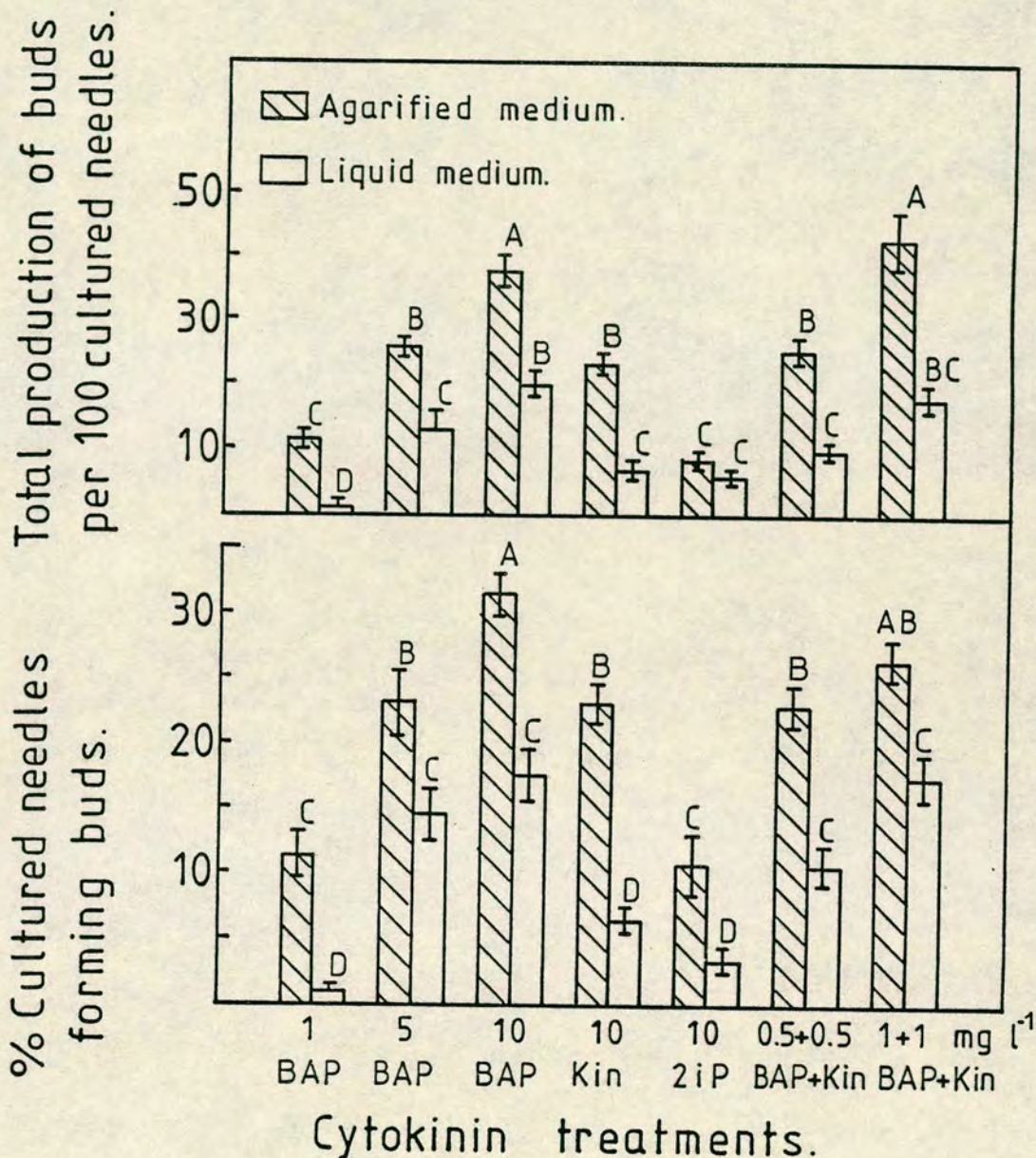


Fig. 3.1.1.14 Effects of the physical properties of modified Schenk and Hildebrandt medium, supplied with various types and levels of cytokinin, on the number and rate of production of adventitious buds on excised needles from seedling of *Pinus brutia* after 8 weeks on the above medium. 280 needles were used for each treatment. Different letters above any two bars indicate that these two means are statistically different at $P=0.001$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

Table 3.1.1.1 Effects of different cytokinin mixtures on the morphogenetic responses of cultured needles from seedlings of *Pinus brutia* after 8 weeks on media containing cytokinins.

Mixture of cytokinins	No of cultured needles	%** needles forming buds	Mean** no. of buds per cultured needle
BAP**+ Kin*	240	20.4 \pm 2.3a	2.2 \pm 0.2a
BAP + 2iP*	240	11.2 \pm 1.7b	1.5 \pm 0.2b
Kin + 2iP	240	2.9 \pm 1.1c	0.3 \pm 0.1c
CV (%)		66	69

* BAP, 6-benzylaminopurine; Kin, kinetin; 2iP, isopentenylpurine

**Means in column not followed by a common letter are statistically different at $P=0.01$ level (Duncan's multiple-range test).

Table 3.1.1.2 Effects of exogenous auxin (NAA) added to a cytokinin-containing medium on the morphogenetic responses of cultured seedling needles of *Pinus brutia* after 8 weeks on medium containing either a cytokinin (CK) and auxin or a cytokinin mixture (CKmx) and auxin.

Treatments	No. of cultured needles	Means* of morphogenetic response		
		% needles forming buds	No of buds per cultured needle	% needles with excess callus
CK	720	12.5 \pm 1.8a	0.83 \pm 0.1a	6.3 \pm 1.4a
CK +auxin	720	9.2 \pm 1.4a	0.78 \pm 0.1a	38.2 \pm 3.3b
CV (%)		70	65	66
CKmx	360	9.7 \pm 1.7a	1.30 \pm 0.2a	8.6 \pm 2.5a
CKmx +auxin	360	13.3 \pm 1.9a	1.30 \pm 0.2a	16.4 \pm 2.5a
CV (%)		66	69	47

*Means in column not followed by a common letter are statistically different at $P=0.001$ level in upper part, and at $P=0.05$ level in lower part

Table 3.1.1.3 Effects of four concentrations of 6-benzylaminopurine (BAP), each applied as pulses for 2 hours duration, on the yield of adventitious buds and frequency of mortality of *Pinus brutia* seedling-needles cultured *in vitro* 8 weeks after application. Following pulse treatment, explants were cultured (and subcultured) on agar medium devoid of cytokinins.

BAP level mg l ⁻¹	No. of cultured needles	%* needles forming buds	Mean* no of buds per cultured needle	%* of mortality
50	300	22.3 ±2.6 a	1.5 ±0.2 a	0.0 ±0.0 a
100	300	7.3 ±1.6 b	0.5 ±0.1 b	0.0 ±0.0 a
200	300	2.9 ±1.3 bc	0.2 ±0.01bc	7.0 ±0.4 b
400	300	1.0 ±0.7 c	0.08 ±0.06c	18.2 ±1.0 c
CV (%)		29	67	29

* Means in column not followed by a common letter are statistically different at $P=0.001$ level (Duncan's multiple-range test).

Table 3.1.1.4 Effects of activated charcoal (AC) on development and elongation of buds induced on cultured seedling-needles of *Pinus brutia* after 6 weeks on half-strength medium with or without AC (1%).

Treatments	No. of induced buds	Mean* height of developed shoot (mm)
medium without AC	150	4.9 ± 0.3 a
medium with AC	150	10.2 ± 0.8 b
CV (%)		32

* Means in column not followed by a common letter are statistically different at $P = 0.001$ level.

induced buds formed) was less when cultured needles were incubated in a liquid medium than on a solid medium, irrespective of the type and concentration of cytokinin applied (Fig. 3.1.1.14).

Callus growth on cultured explants was promoted more in a liquid than on a solid medium (grand means of 28.2 ± 1.6 versus 14.2 ± 1.2 respectively). Explants left in a liquid medium for a period longer than 3-4 weeks turned brown. Vitrified buds and shoots also appeared in 21% of the cultured needles in liquid medium. The shoots turned translucent with clear succulent green needles that adhered to each other.

iv. Influence of age of donor seedlings

The potential for inducing adventitious buds on cultured needles declined significantly with increasing age of the explanted seedlings (Fig. 3.1.1.15). This general result was not affected by the concentration of BAP applied (5 and 10 mg l⁻¹, in which the corresponding percentage of needles forming buds was 32.2 ± 1.8 and 37.7 ± 2.1 respectively).

B. Multiplication of induced adventitious buds

i. Influence of cytokinin treatments

The results shown in Fig. 3.1.1.17 clearly reveal that the frequency of cultured needles forming buds remained constant after an induction-time of 8 weeks on CK-containing medium for the direct-cultured explants, and likewise on CK-free medium for pulse treated explants. A further incubation time, in both cases, led to an increase in number of induced buds per explant. However, the rate of

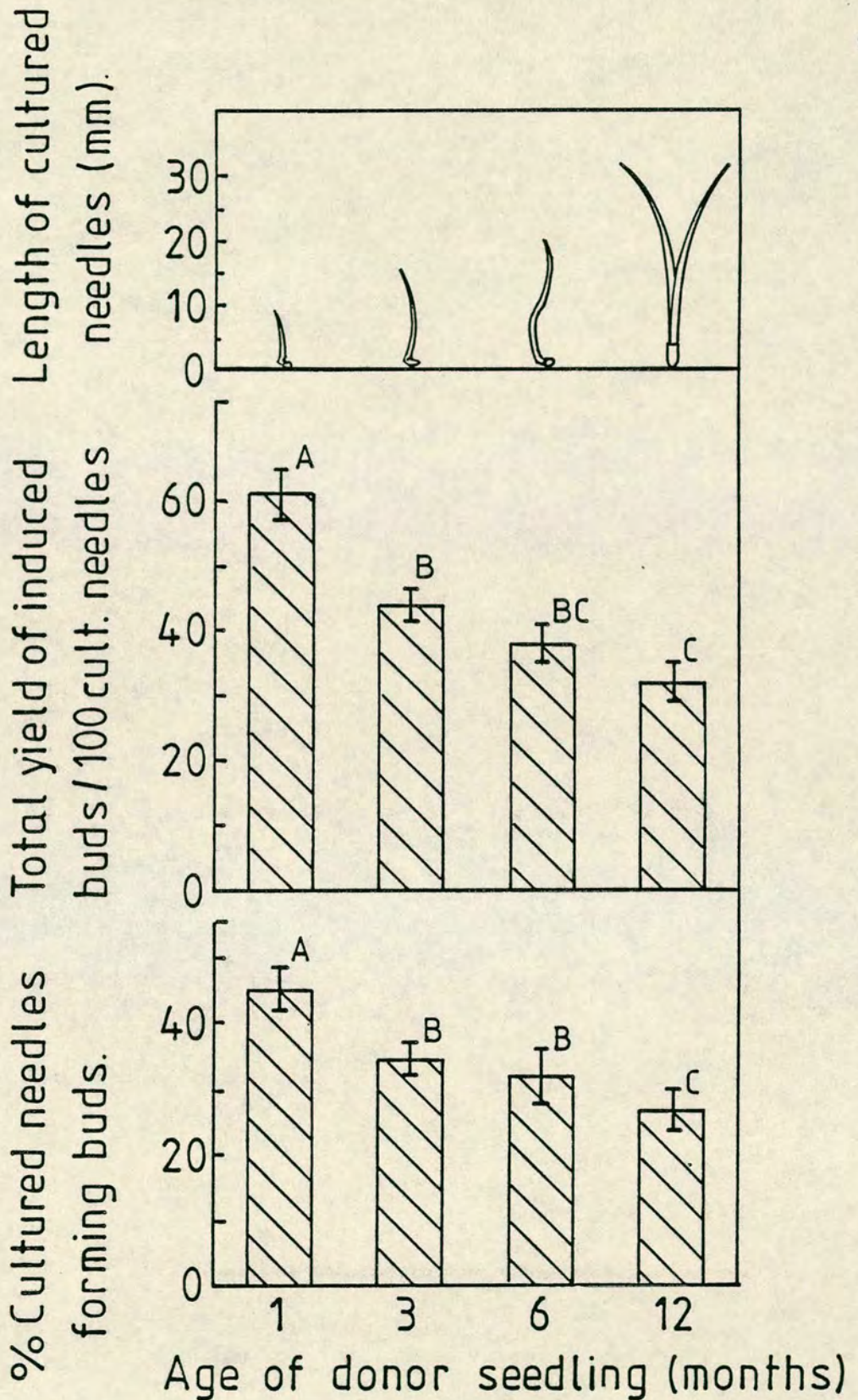


Fig. 3.1.1.15 The production of adventitious buds on cultured needles of *Pinus brutia* excised from seedlings of different age-classes. Explants were cultured for 8 weeks on medium containing cytokinin. 360 needles were used for each treatment. Different letters above any two bars indicate that these two means are significantly different at $P=0.001$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

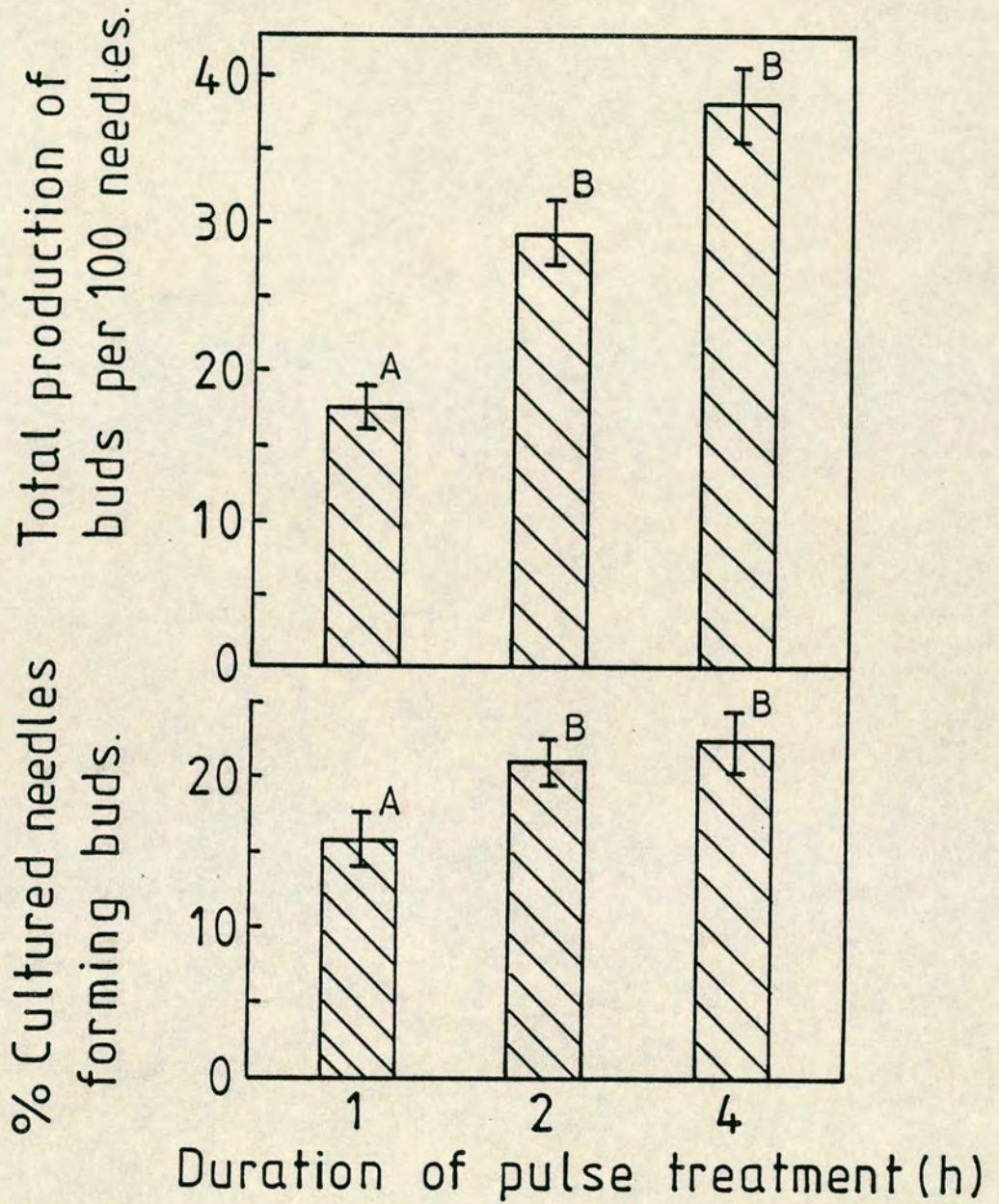


Fig. 3.1.1.16 Effects of duration of the treatment at 50 mg l^{-1} BAP on the production of adventitious buds on cultured needles of *Pinus brutia*. Explants were then cultured on BAP-free medium for 8 weeks. 360 explants were used for each treatment. Different letters above any two bars indicate that these two means are statistically different at $P=0.001$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

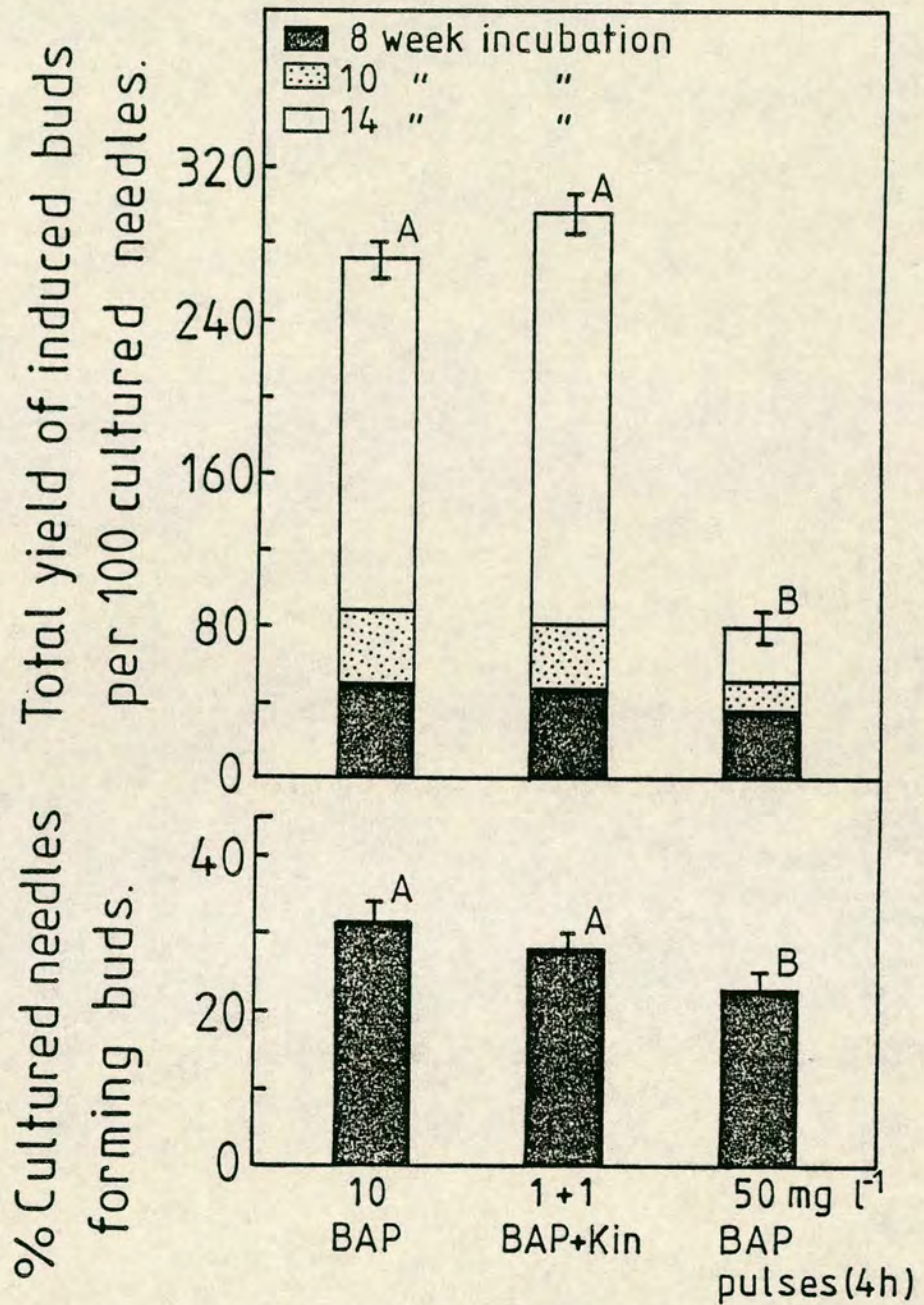


Fig. 3.1.1.17 Effects of mode of application of exogenous cytokinins on the production of adventitious buds on *Pinus brutia* needles cultured in vitro after 8, 10, and 14 weeks. Explants were either pulse treated with 50 mg l⁻¹ BAP for 4 h before culture on CK-free medium, or directly cultured on CK-containing medium. 360-720 explants were used for each treatment. Different letters above any two bars indicate that these two means are significantly different at P= 0.001 level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

this increase over a longer period of 14 weeks was found to be higher on the directly cultured explants and then sub-cultured on CK-containing medium, rather than on the pulse treated explants. The best result was achieved, in the former case, on a medium supplied with a mixture of BAP + kin (each at a level of 1 mg l^{-1}) (Fig. 3.1.1.12 and 3.1.1.17).

C. Shoot formation from induced adventitious buds

i. Influence of activated charcoal

Induced adventitious buds sub-cultured onto a medium without activated charcoal (AC) did not grow beyond a mean height of 4.9 mm; however, the growth of buds which had been transferred onto another medium with 1% AC was considerably promoted and elongation was more than 10.2 mm within 4-6 weeks (Table 3.1.1.4).

D. Final account

A final tally of the production of adventitious buds on cultured needles (excised from 8 week-old seedlings) was 272 and 296 buds per 100 cultured needles, after a total incubation time of 14 weeks, on media with 10 mg l^{-1} BAP and $1 + 1 \text{ mg l}^{-1}$ of BAP + kin respectively (Fig. 3.1.17). Following the method developed for bud elongation into shoots on AC-containing medium for 4 weeks (Table 3.1.1.4), about 81 % of the induced buds developed into normal shoots (>10 mm in height). Accordingly, shoot production in the range of 44-48 per seedling (20 needles used from each seedling) was achieved within a total period of 18 weeks.

3.1.2 AXILLARY SHOOT FORMATION ON SEEDLING-SHOOT EXPLANTS

Axillary shoot production is perhaps the most promising of all the micropropagation techniques applicable to conifers. It involves the activation of axillary buds which already exist in the leaf axils, but which are normally held in check by apical dominance.

In this section, the potential of this technique is explored using seedling material of *Pinus brutia*. Factors influencing the activation of axillary buds, and their subsequent development have also been examined.

A. Induction and development of axillary buds and shoots

i. Anatomical observations with the light and scanning

electron microscope

The buds which became prominent during culture arose from existing buds in the axils of some juvenile needles on the shoot axis (Fig. 3.1.2.1). At explantation these buds were at an early stage of development (Fig. 3.1.2.2 and 3.1.2.3), and grew out within one week after placing on a modified SH medium containing cytokinin (Fig. 3.1.2.4 and 3.1.2.5). Only another 1-2 weeks were required to complete development of these structures into vigorous buds (Fig. 3.1.2.6). Further development of these buds into normal shoots was associated with the appearance of clusters of secondary buds at the bases of the primary buds (Fig. 3.1.2.7) after a total incubation time of 6 weeks on the same medium.

ii. Shoot formation from primary axillary buds

In general, the frequency of explants that formed buds was 98-100%.

Fig. 3.1.2.1-3.1.2.8: Scanning electron micrographs and light micrographs of the developmental sequence of immature buds. These buds already present on shoots from seedlings of *Pinus brutia* developed into vigorous axillary buds and shoot after 6 weeks of culture on a modified SH medium supplied with 3 mg l^{-1} BAP. Fig. 1. Calabrian pine seedling with an axillary bud (arrow). Explant before culture. Scale bar= 0.5 mm. Fig. 2. Scanning electron micrograph of the same stage as in Fig. 1 showing the bud enclosed within bud scales (arrow). Scale bar= 100 μm . Fig. 3. Median longitudinal section of the an early stage with leaf primordia (black arrow) and bud scales (open arrow) Scale bar = 1 mm. Fig. 4. Scanning electron micrograph of the same structure after 1 week on a medium with cytokinins, showing how the bud scales have started to open (arrow). Scale bar= 100 μm . Fig.5. Median longitudinal section of the same structure as in Fig. 4 showing a developed primordium. Scale bar= 1 mm. Fig. 6. Median longitudinal section of a well-developed bud after 2-3 weeks on the culture medium. Scale bar= 1.5 mm. Fig.7 Primary axillary shoot formation (black arrows) with masses of secondary buds (white arrows) on cultured explants after 6 weeks on cytokinin medium. Scale bar = 2.5 cm. Fig. 8. Production of primary axillary shoots (arrows) on two types of cultured explants (a) shoot explant without an apical bud; (b) shoot explant with an apical bud. Scale bar = 2 cm.



However, yields of buds and shoots per explant and their appearance varied markedly according to the treatments applied (Table 3.1.2.1 and 3.1.2.2). After 6 weeks on the shoot-forming medium (with cytokinin), the maximum number of shoots produced per explant was obtained with 3 mg l⁻¹ of BAP or the BAP + kin mixture (Table 3.1.2.1). Removal of the apical bud increased the yield of primary shoots (Fig. 3.1.2.8), particularly in conjunction with BAP + kin (Fig. 3.1.2.9).

iii. *Formation of secondary buds*

Masses of secondary buds could be induced at the base of each primary shoot, after the initial period of incubation (6 weeks) (Fig. 3.1.2.7). Surgical removal of the apical bud significantly increased the production of secondary buds compared with the intact explants (Table 3.1.2.2).

B. *Shoot-bud multiplication*

The production of shoots from secondary buds, after 12 weeks under shoot-elongated conditions (without added cytokinin), was higher on cultured explants that initially received low levels of cytokinin than on those initially incubated on a higher level of BAP (5 mg l⁻¹) (Fig. 3.1.2.10). When individual primary shoots were maintained on a medium with BAP + kin, 2-3 new buds were induced at the axil, and after each of the new buds had elongated into a shoot, this shoot was again exposed to cytokinins for the induction of further crop. Accordingly, two crops of shoots were produced successively each within 4-6 weeks.

Table 3.1.2.1 Effect of two levels of 6-benzylaminopurine (BAP) and a mixture of two cytokinins (BAP and kinetin (Kin)) on the production of primary axillary shoots and secondary buds on cultured shoot explants of *Pinus brutia* Ten. after 6 weeks on a modified Schenk and Hildebrandt medium (Abdullah et al. 1985).

Level of cytokinin mg l ⁻¹	Mean* no. of primary shoots per explant	Mean* height (mm) of primary shoot	No. of secondary buds per explant
BAP (3)	2.3 ±0.1a	7.8 ±0.3a	6.5 ±0.5a
BAP (5)	1.0 ±0.09b	4.3 ±0.4b	4.5 ±0.4b
BAP (1)+ Kin (1)	2.3 ±0.1a	8.8 ±0.3a	4.6 ±0.3b
CV(%)	35	37	51

* Means in columns not followed by a common letter are statistically different from each other at $P = 0.01$ level (Duncan's multiple-range test).

Table 3.1.2.2 Effect of excision of the apical bud from cultured shoot explants of *Pinus brutia* Ten. on the production of primary axillary shoots and secondary buds after 6 weeks on a modified Schenk and Hildebrandt medium supplemented with cytokinin.

Types of cultured explant	Mean* no. of primary shoots per explant	Mean* height (mm) of primary shoot	Mean no. of secondary buds per explant
shoot without apical bud	2.3 ±0.1a	7.6 ±0.3a	7.2±0.3a
shoot with apical bud	1.4 ±0.1b	6.4 ±0.4b	3.2±0.1b
CV(%)	35	37	51

* Means in columns not followed by a common letter are statistically different from each other at $P = 0.01$ level.

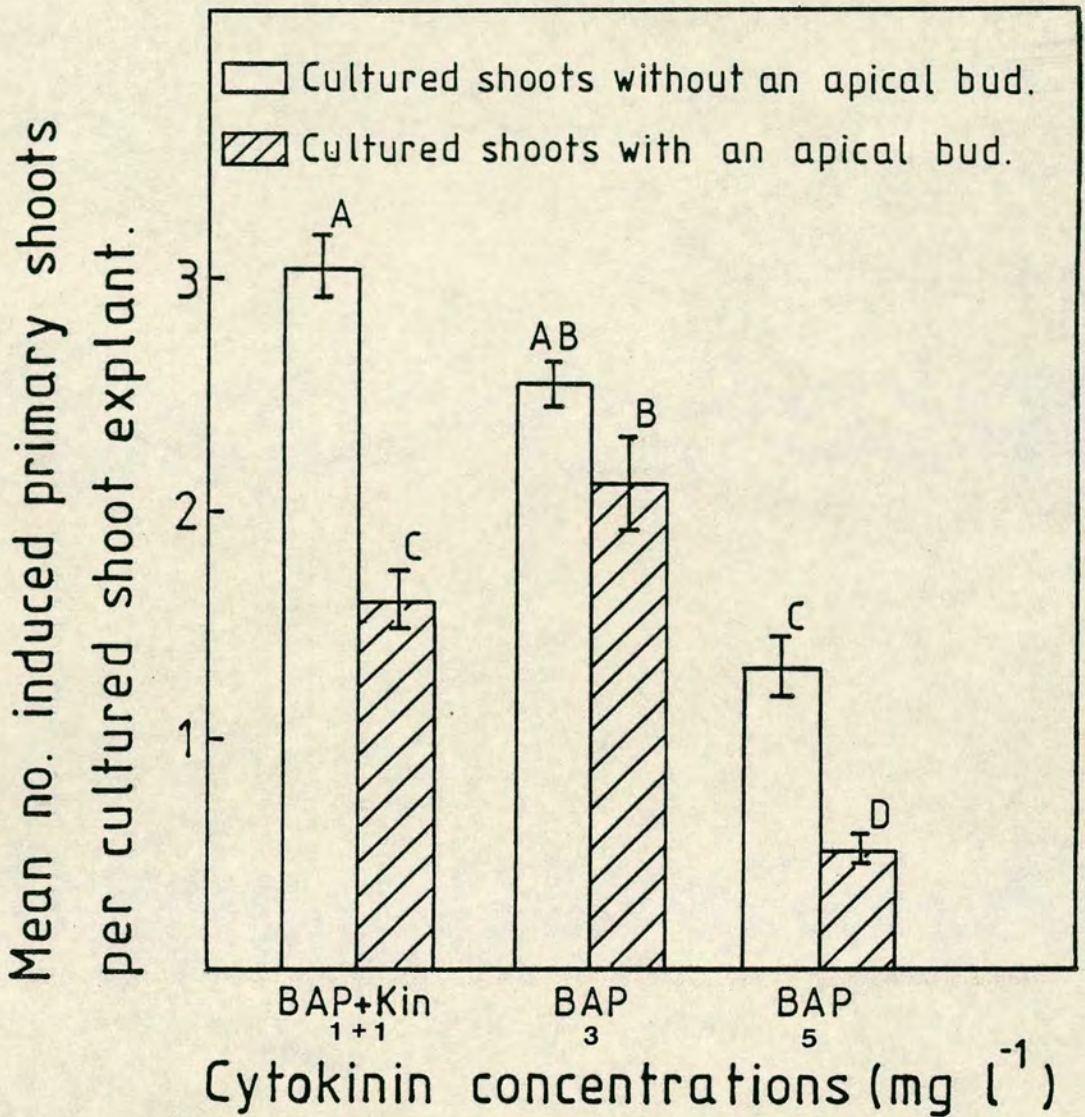


Fig. 3.1.2.9 Interaction of the applied cytokinin level with the presence or absence of the apical bud from the culture shoots on the yield of primary axillary shoots per cultured shoots of *Pinus brutia* Ten. after 6 weeks on a modified SH medium. 60 explants were used for each treatment. Different letters above any two bars indicate that those two means are significantly different at $P=0.001$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

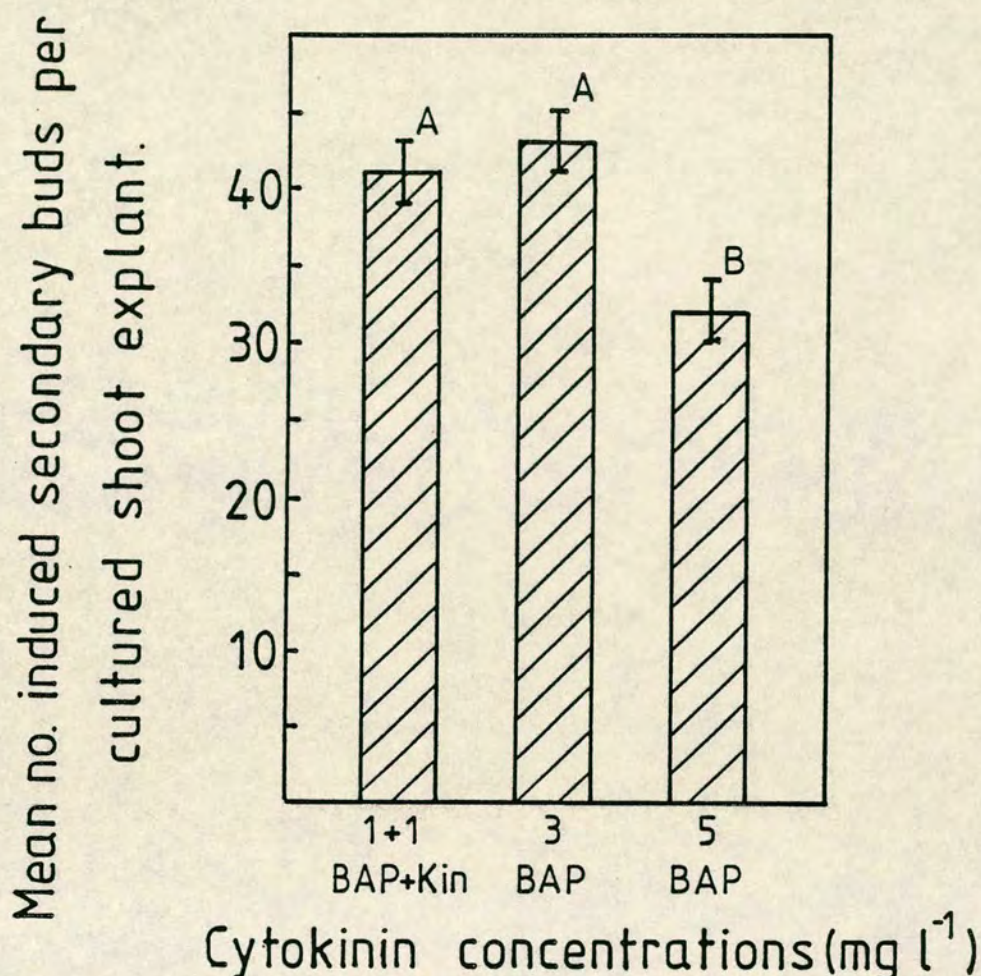


Fig. 3.1.2.10 Effects of different cytokinin treatments, initially applied for 6 weeks for the production of primary axillary shoots and secondary buds on decapitated shoot from seedlings of *Pinus brutia*, on subsequent yields of secondary buds on the cultured explants after another 12 weeks on cytokinin-free medium. Different letters above any two bars indicate that these two means are significantly different at $P=0.01$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

C. Shoot elongation

Shoot elongation (>20 mm in height) was achieved on both half-strength SH medium without cytokinin, and on the latter medium containing 1 % activated charcoal. However, the presence of activated charcoal decreased the necessary incubation time required from 4-6 weeks to 2-3 weeks.

D. Axillary shoot formation on explants of

shoots from 6-month-old seedlings

The procedure described above induced 98% of the explants to develop axillary buds, when applied to explants from 6 month-old seedlings. These subsequently developed into vigorous shoots when placed in shoot-elongating conditions.

3.1.3 FORMATION OF SHOOT-BUDS ON MATURE FASCICLE NEEDLES

It is generally considered that as a tree approaches maturity and becomes old enough to manifest its desirable qualities, it also becomes very difficult to propagate *in vitro*. Morphogenic responses of explants from mature trees, particularly conifers, are often hard to obtain, apparently because of the susceptibility of these tissues once excised to oxidative browning due to their high content of hydroxyphenols. It was necessary, therefore, using various approaches to attempt to overcome these problems with a view to using cultured fascicles of *Pinus brutia* for the mass clonal propagation of mature trees.

3.1.3.1 Initiation of shoot-buds

A. Influence of anti-browning substances

i. Rate of oxidative browning on cultured fascicles

Frequency of browning of cultured fascicles was affected differently by the range of treatments applied. Soaking the fascicles in water for a short time (1 h) was insufficient to leach out the inhibitory phenols, and soaking for a longer time (2 h and up to 24 h) increased the amount of browning (Fig. 3.1.3.1). Using the other soak treatments resulted in two general effects: soaking in high concentrations for a short time (15-60 min.) in any of the solutions nearly always caused substantial browning, while soaking for a longer duration (up to 24 h) at low concentrations of these substances (especially IBA and PPZ) reduced the phenolic browning (Fig. 3.1.3.1). Browning was also significantly reduced either by adding insoluble polyamides (PVP), or chelate (SDD) to the culture medium (Fig. 3.1.3.1). With the SDD and

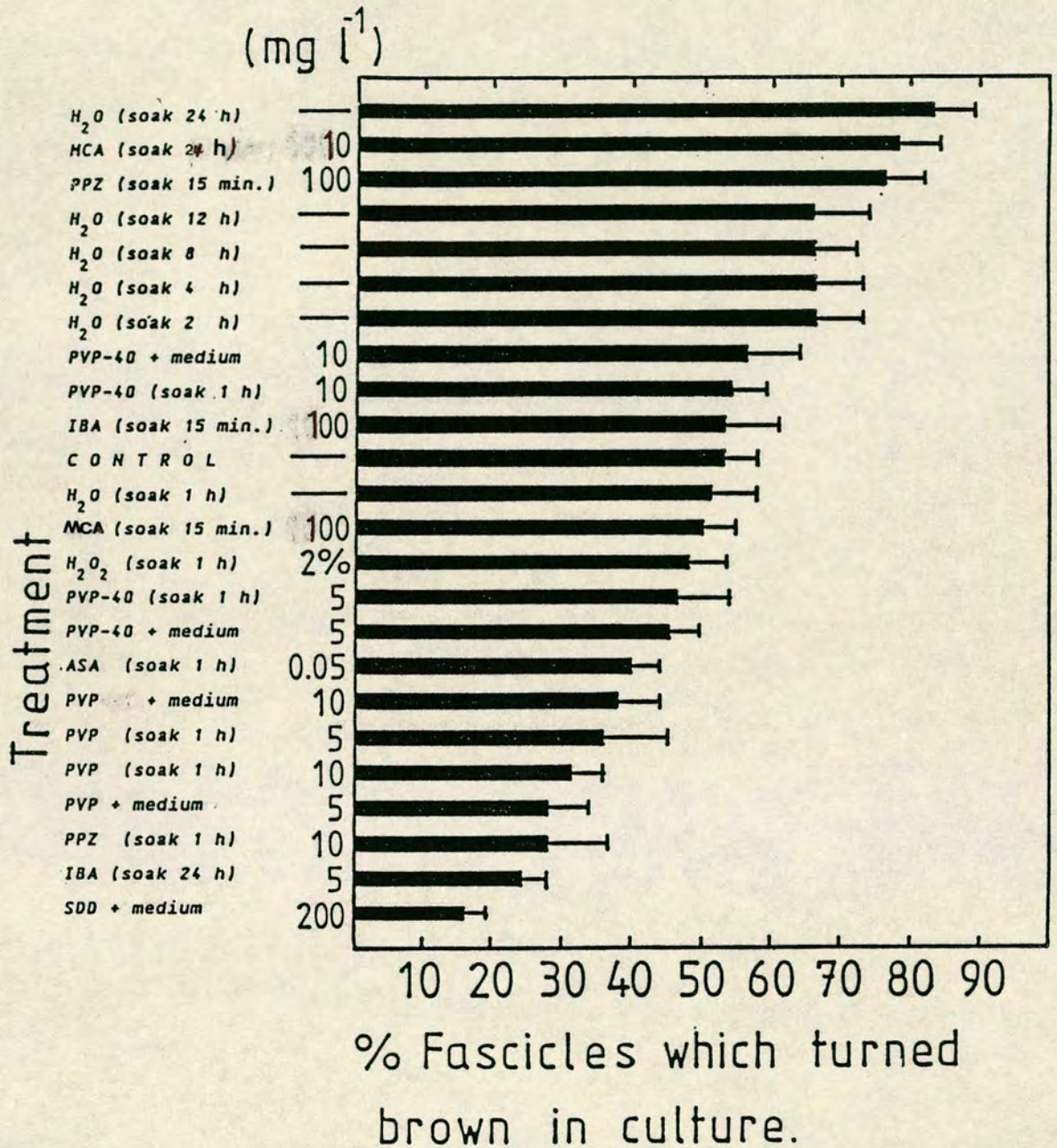


Fig. 3.1.3.1 Effects of different treatments designed to reduce the susceptibility of cultured fascicles of 10 year-old trees of *Pinus brutia* to browning. Treatments were either applied as a soak solution before culture or included in the culture medium for 10 weeks. Treatments were significantly different at P=0.001 level (Duncan's multiple-range test). Horizontal bars represent the standard error of the mean.

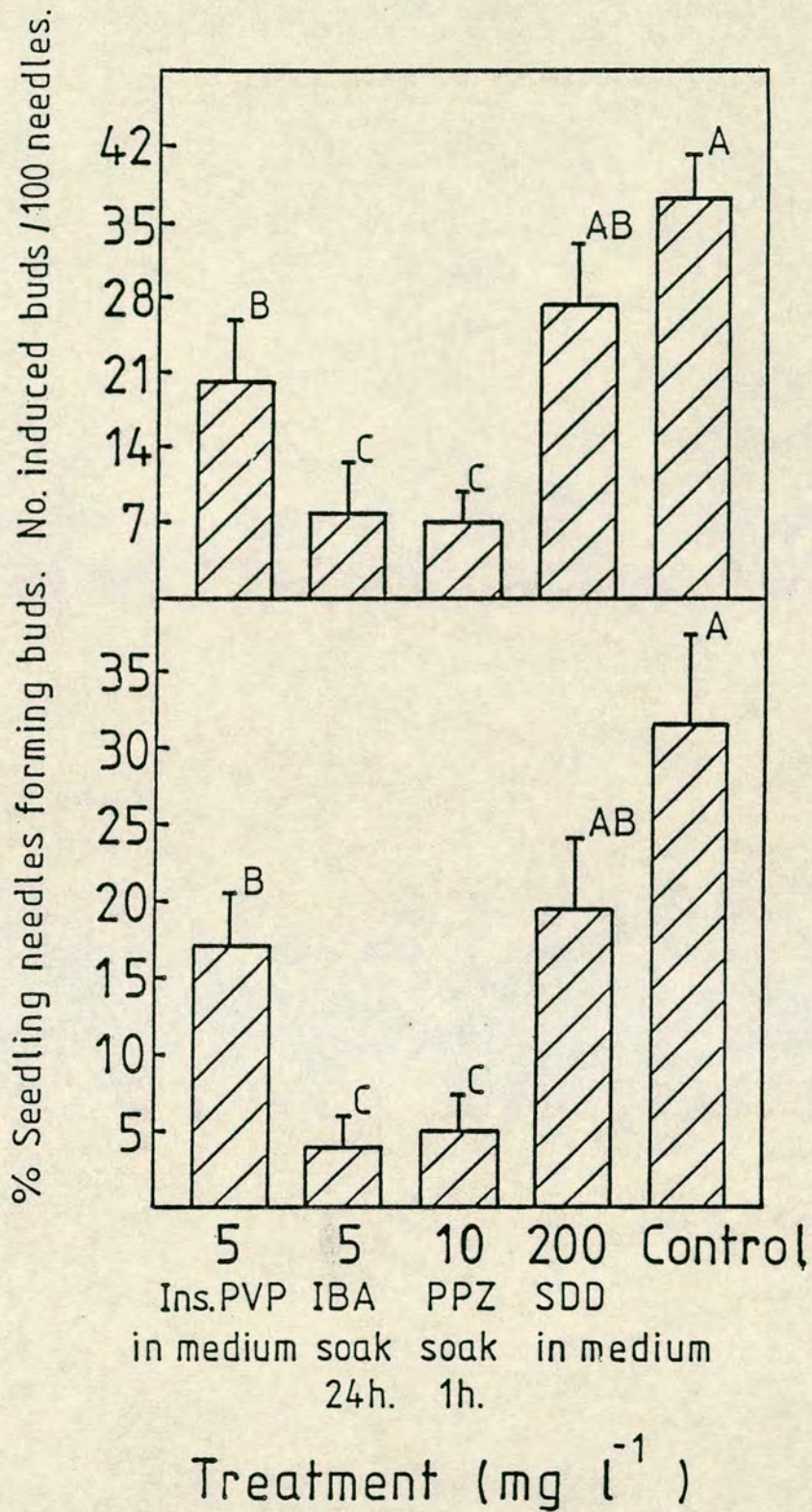


Fig. 3.1.3.2 Effects of the best four treatments, previously shown to prevent browning of cultured needles of 10 year-old *Pinus brutia*, on the developmental response of cultured seedling-needles. Treatments were applied either as soak solutions before culture or included in the culture medium for 10 weeks. Different letters above any two bars indicate that these two means are significantly different at $P=0.001$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

insoluble PVP treatments shoot-buds were activated on the cultured fascicles, whereas soaking in IBA solutions (5 or 1000 mg l⁻¹) caused excess callus proliferation at the base of the cultured explants.

ii. Morphogenic responses of excised needles from seedlings

'Chelate' (SDD, 200 mg l⁻¹) was the only additive which supported a high incidence of bud induction on the cultured seedling-needles (Fig. 3.1.3.2). However, the results of further investigations revealed that the addition of SDD at a concentration of 150 mg l⁻¹ to the culture medium was more effective in supporting an enhanced production of adventitious buds than at a level of 200 mg l⁻¹ (Fig. 3.1.3.3). This treatment was applied in the following experiment.

B. Influence of season of collection}

i. Rate of oxidative browning of cultured fascicles

Intensity of browning of fascicles collected in June, on an SDD-containing medium, was found to be greater than with material sampled in July (Table 3.1.3.1).

ii. Morphogenic responses of cultured fascicles

Fascicles explanted in July (Fig. 3.1.3.5, b) were more responsive than those sampled in June (Fig. 3.1.3.5, a), in terms of their capacity to form callus and shoot-buds (Table 3.1.3.1). The latter time of collection was adopted in the following experiments.

C. Influence of sequential treatments

A sequence of SDD treatment (150 mg l⁻¹ for 6 weeks) and insoluble PVP treatment (1 mg l⁻¹ for further 4 weeks) appeared to be advantageous in increasing the number of shoot-buds produced and in eliminating browning (Fig. 3.1.3.4). The pretreatment of explants for 2 weeks on 1% activated charcoal was not effective in reducing

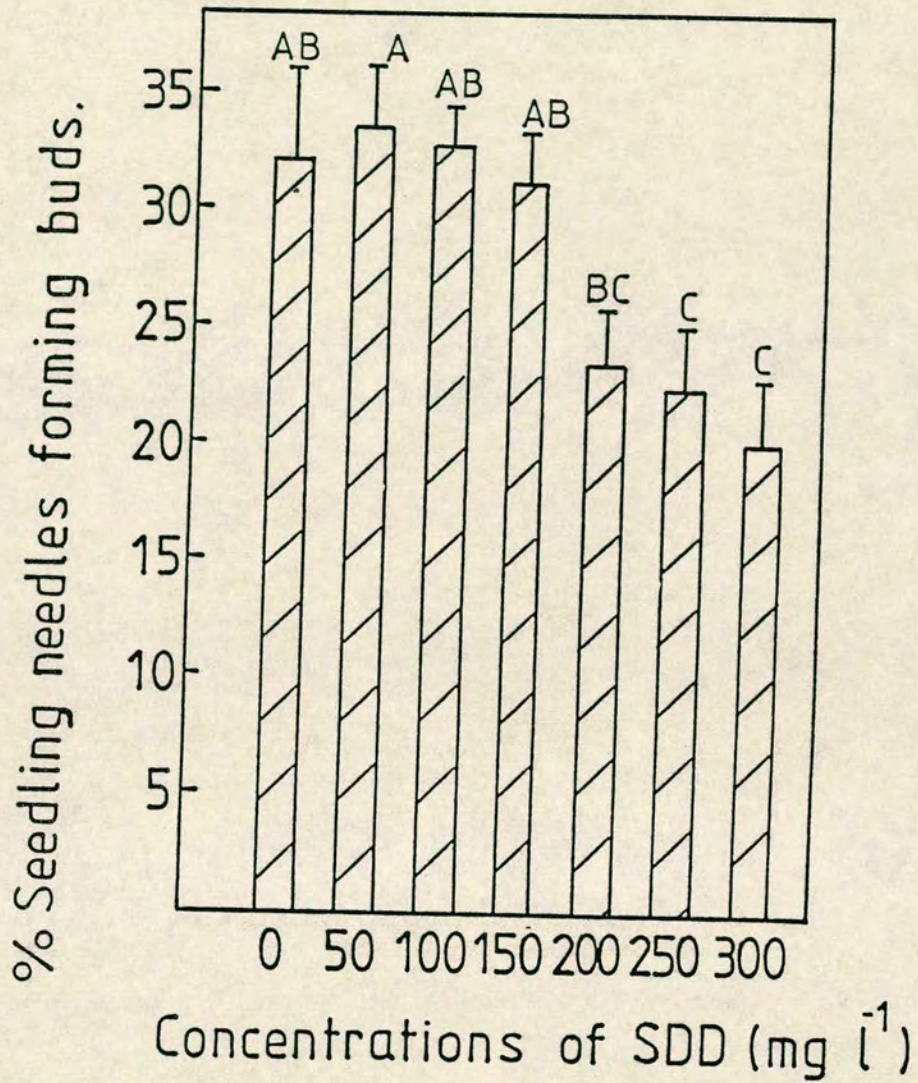


Fig. 3.1.3.3 The production of adventitious buds on cultured needles excised from seedlings of *Pinus brutia* 10 weeks after incubation on a cytokinin-containing medium supplemented with various concentrations of the chelate (sodium diethyldithiocarbamate, SDD). Different letters above any two bars indicate that these two means are significantly different at $P=0.001$ level (Duncan's multiple-range test) Vertical bars represent the standard error of the mean.

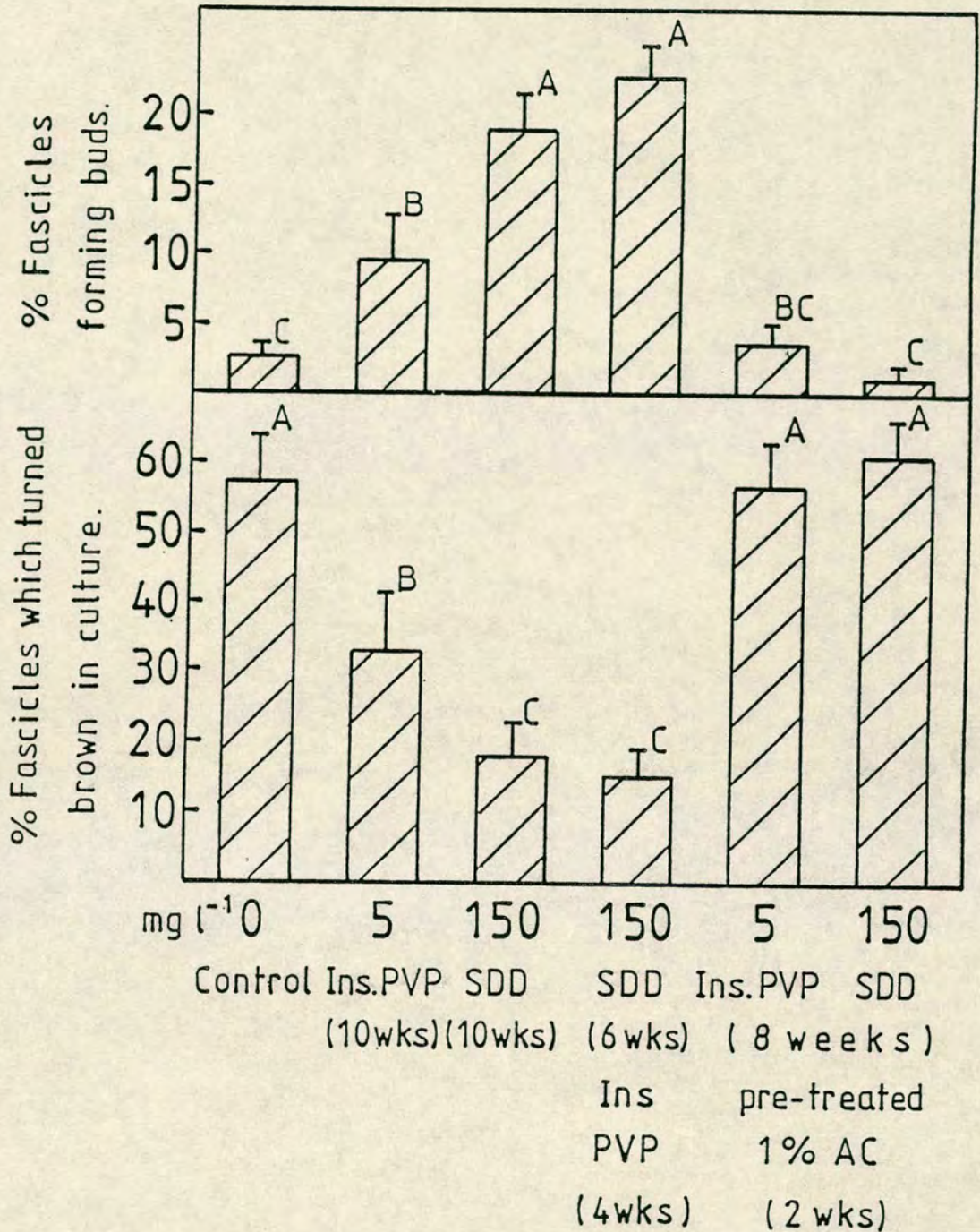
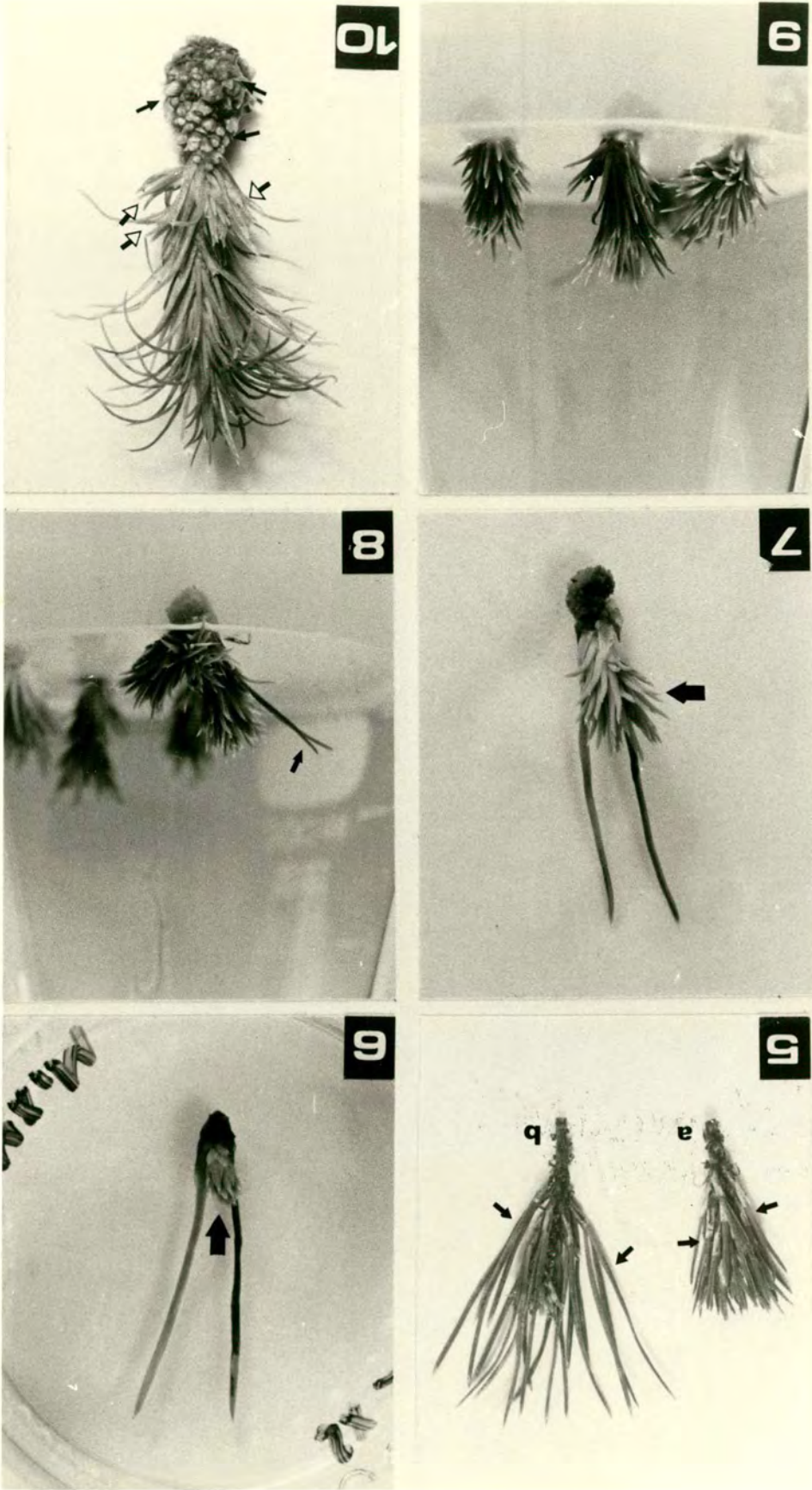


Fig. 3.1.3.4 Frequency of shoot-bud production and the proportion of browning on cultured fascicles of 10 year-old trees of *Pinus brutia*. Explants were cultured for 10 weeks on a cytokinin-containing medium supplied with various types and levels of additives for different incubation-times. Different letters above any two bars indicate that these two means are significantly different at $P=0.001$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

Fig. 3.1.3.5--3.1.3.10 Formation of shoot-buds on cultured fascicle needles of 10 year-old trees of *Pinus brutia* on a modified Schenk and Hildebrandt medium containing a mixture of BAP+Kin (5 mg l^{-1} for each) and supplemented with chelate (sodium diethyldithiocarbamate at level of 150 mg l^{-1}). Fig. 5. Initial explants were fascicles (arrow) collected either in a, June or b, July. Fig. 6. The development of shoot-bud (arrow) in the axil of the cultured fascicles 10 weeks after incubation on the initiation medium. Fig. 7. Elongation of the induced shoot-bud (arrow) after another 4 weeks on a medium supplied with a mixture of BAP+Kin (1 mg l^{-1} for each). Fig. 8. An early growth of fascicle needles (arrow) on the elongated shoot-buds after a total incubation of 10 weeks on the later medium. Fig. 9. Elongation of induced shoot-bud after 10 weeks of incubation on cytokinin-free medium. Fig. 10. Multiplication of elongated shoot-buds after further 8 weeks incubation on a medium included a mixture of BAP+Kin (2.5 mg l^{-1} for each). Note the induction of new crop of shoots (white arrows) and cluster of buds (black arrows) at the shoot base.



browning, but did lead to some elongation of the basal region of the cultured fascicles (Fig. 3.1.3.4).

3.1.3.2 Elongation of induced shoot-buds

Induced shoot-buds (Fig. 3.1.3.6), incubated for 4 weeks on a diluted modified SH medium containing a low level of a cytokinin mixture, exhibited a significant enhancement in elongation (Table 3.1.3.2 and Fig. 3.1.3.7). Whereas, further incubation beyond 4 weeks and up to 10 weeks on the above medium tended to limit further elongation. Such treatment rarely stimulated early growth of fascicles on the elongated shoots, (Table 3.1.3.2 and Fig. 3.1.3.8). Alternatively, a long term incubation of induced shoot-buds (Fig. 3.1.3.6) for 10 weeks on a diluted medium devoid of cytokinins caused a considerable increase in shoot elongation (Table 3.1.3.2 and Fig. 3.1.3.9).

3.1.3.3 Multiplication of elongated shoot-buds

A high rate of growth in height and an advanced overall development was displayed by the induced shoot-buds, 10 weeks after incubation on the initiation medium and similarly on elongation-medium. This was crucial for attaining an effective multiplication rate of these shoots. Elongated shoots equal to or > 20 mm in height were capable of producing further crops of shoots (2-6 per individual) and buds (15-100 per individual) after another 8 weeks on a medium designed for this purpose (Table 3.1.3.3 and Fig. 3.1.3.10).

Table 3.1.3.1. Effect of season of collection of fascicles from 10 year-old *Pinus brutia* trees, on the frequency at which the cultured fascicles form buds and callus. Also the intensity of browning on the fascicles after 10 weeks culture on a modified Schenk and Hildebrandt medium supplied with BAP+Kin (5 mg l⁻¹ of each) and 150 mg l⁻¹ of sodium diethyldithiocarbamate (SDD).

Time of collection 1984	No. of cultured fascicles	% of fascicles forming callus	% of fascicles forming buds	% of fascicles which turned brown
Mid- June	180	34.4 ±2.2 a	4.4 ±1.4 a	30.0 ±1.9 a
Mid- July	180	47.2 ±2.4 b	17.2 ±2.1 b	17.8 ±2.2 b
CV (%)		32	41	52

Means in columns not followed by a common letter are statistically different from each other at $P= 0.001$ level.

Table 3.1.3.2 Effects of cytokinins (CK) on development and elongation of shoot-buds induced on cultured fascicles of *Pinus brutia*. Induced shoot-buds were maintained for incubation times of 4 and 10 weeks on half-strength of a modified Schenk and Hildebrandt (SH) medium either with or without BAP+Kin (1 mg l⁻¹ of each).

Media	Total no. cultured shoot-buds	4 weeks incubation	10 weeks incubation
		Mean* height (mm)	Mean* height (mm)
1/2 SH+ CK	45	10.1 ± 0.20 a	12.9 ± 0.5 a
1/2 SH	45	8.1 ± 0.15 b	21.9 ± 0.8 b
CV (%)		12.1	21.8

* Means in columns not followed by a common letter are statistically different from each other at $P= 0.001$ level.

Table 3.1.3.3 Effect of shoot height and stage of development on induction shoot-buds which have been grown on fascicles of *Pinus brutia* on their subsequent capability for multiplication after they have been cultured for 8 weeks on modified Schenk and Hildebrandt medi supplied with a mixture of BAP+Kin (2.5 mg l^{-1} of each).

Height of elongated shoot (mm)	Total no. of elongated cultured shoots	No. of shoots produced/cultured elongated shoot			No. of buds produced/cultured elongated shoot		
		Min.	Max.	Mean *	Min.	Max.	Mean *
		10-- 15	30	0.0	1.0	0.3 ± 0.1 a	0
20-- 28	30	2.0	6.0	3.8 ± 0.5 b	15	100	55 ± 6.7 b
CV (%)				68			67

* Means in column not followed by a common letter are statistically different from each other at $P= 0.001$ level.

3.2. THE REGENERATION AND ESTABLISHMENT OF PLANTLETS

In this part, the results are presented of a study into the formation of plantlets using adventitious and axillary-derived shoots produced on various explants from shoots of *Pinus brutia*. In particular the efficiency of the methods developed, for future cloning and vegetative propagation of this species, was examined in relation to two major criteria:-

1. The rate and quality of the adventitious root systems induced and the corresponding growth of the shoots.
2. The survival and performance *in vivo* of the regenerated plantlets compared with normal seedlings.

In section 3.2.1, the experiments were directed towards the development of a sequential treatment, mainly using exogenous growth substances, to produce uniform propagules, developed from cultured axillary buds. The aim was to produce plantlets in which the root systems will ^{be} highly branched, possess a well developed mycorrhizal association, and balanced growth between root and shoot. Once a suitable protocol had been established, it was applied to the rooting of shoots produced on seedling-needles and those induced on explanted fascicles. Efforts were also made to overcome problems associated with rooting in sterile-agar medium. By using tap water with axillary derived shoots under non-sterile conditions as an alternative root induction medium. In section 3.2.2, investigations are described into the influences of explant origin (adventitious or axillary) on the subsequent pattern of growth and performance *in vivo* of plantlets compared with seedlings. Efforts were aimed at determining if the differences between the growth of the three types of plants (seedling, adventitious or axillary) could be attributed to the poor performance

of the shoot or root systems or to other physiological disorders in the plantlets.

3.2.1 REGENERATION OF PLANTLETS

I. Adventitious root formation on an axillary-derived shoot

The establishment of an effective and uniform root system on shoots grown *in vitro* is critical to the mass production of clonal material for commercial purposes. In a series of experiments, attention has been directed towards the development of an efficient procedure to fulfill the above objective.

A. Influence of various types and concentrations of growth substances

i. Induction of adventitious roots

The cultured shoots exhibited various rooting responses according to the NAA/BAP treatments applied (Fig. 3.2.1.1). With increasing NAA concentration (up to 1.0 mg l^{-1}) the frequency of shoots forming roots increased significantly. However, in the presence of BAP at a low level (0.05 mg l^{-1}) the percentage of shoots forming roots was sharply enhanced at NAA concentrations of 0.5 and 1.0 mg l^{-1} (Fig. 3.2.1.1). NAA alone was more effective in root induction than IBA (Fig. 3.2.1.2). A mixture of two auxins (NAA+IBA) promoted rooting more effectively than any one auxin (Fig. 3.2.1.1 and 3.2.1.2). A mixture of $1+2+0.05 \text{ mg l}^{-1}$ of NAA+IBA+BAP respectively was required to elicit the maximum average rooting response of 73% (Fig. 3.2.1.2), with up to 100% in some clones.

ii. Rate of root elongation and the number of roots produced

Six months after transplanting from agar to soil, there was no adverse after-effect of the inclusion of a low level of BAP (0.05 mg l^{-1}) in the original rooting medium, but there was an indication of an

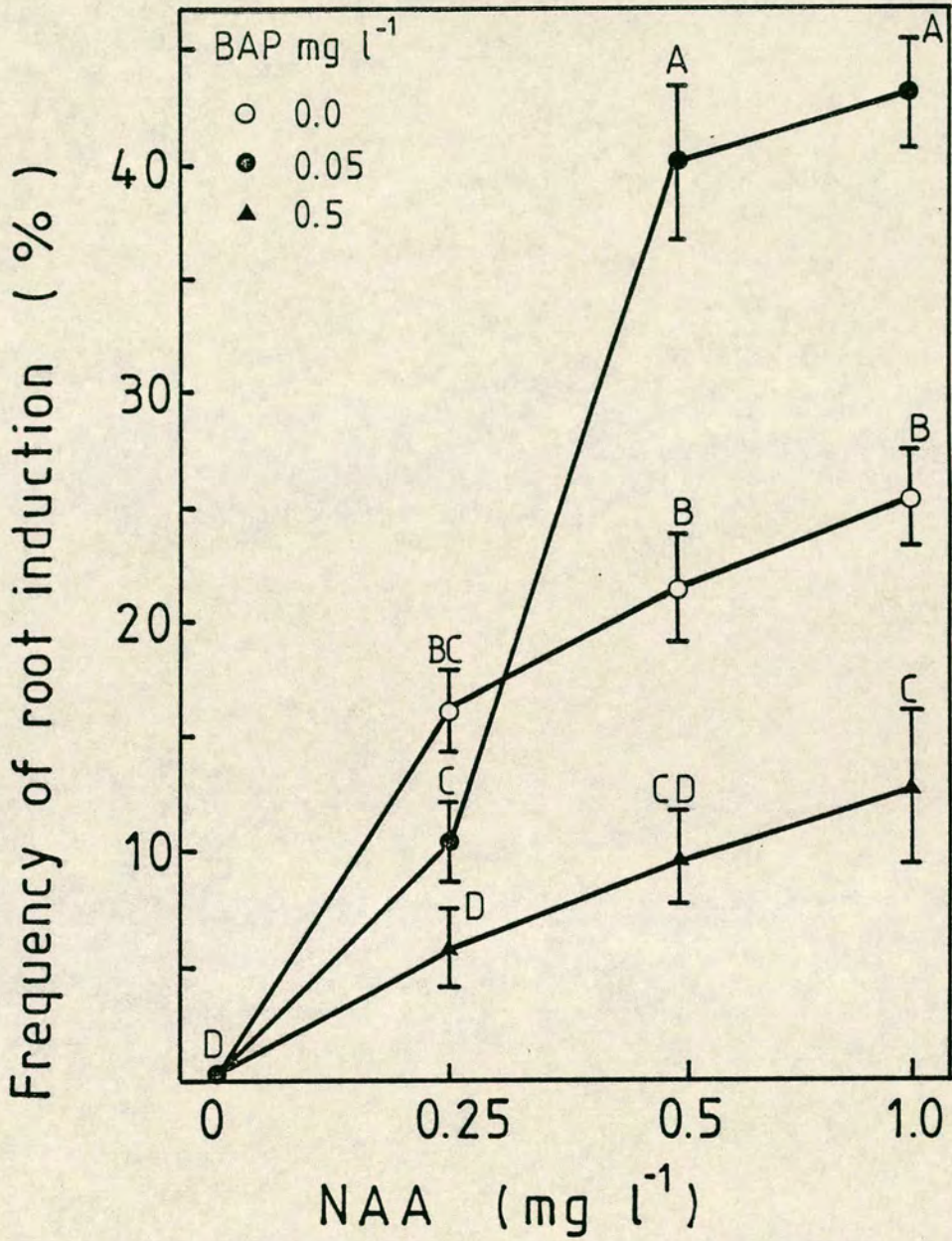


Fig. 3.2.1.1 Interaction of NAA and BAP on the frequency of root induction on axillary shoots of *Pinus brutia*. 90 shoots were used for each combination. Different letters above any two bars indicate that these two means are statistically different at $P = 0.01$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

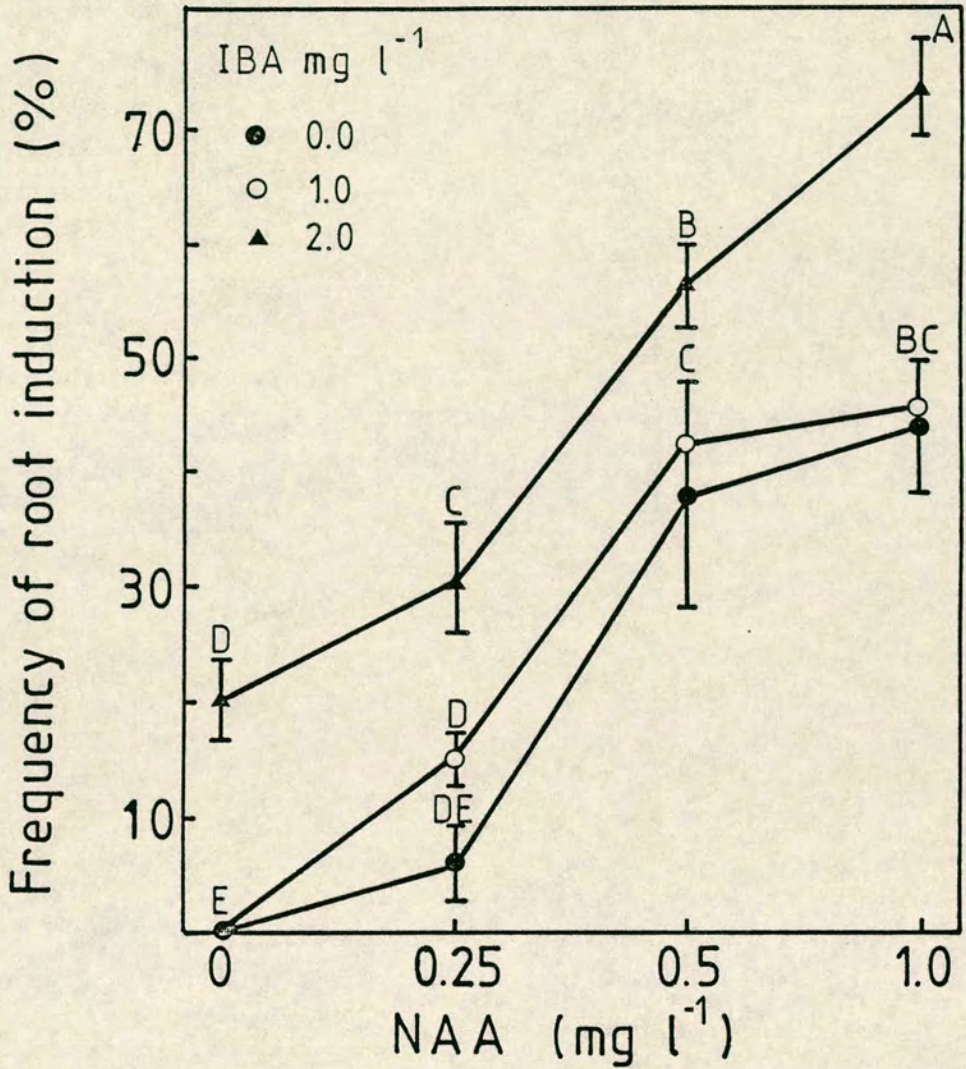


Fig. 3.2.1.2 Interaction of NAA and IBA (all with 0.05 mg l⁻¹ BAP) on the frequency of root induction on axillary shoots of *Pinus brutia*. 45 shoots were used for each combination. Different letters above any two bars indicate that these two means are statistically different at P= 0.01 level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

increase in the rate of both root and shoot growth (Fig. 3.2.1.3). Whereas, a high concentration of BAP (0.5 mg l^{-1}) had a negative influence on the number of roots per plantlet and the rate of root growth (Fig. 3.2.1.3). The elongation rate of induced roots was also influenced by the interaction between the two auxins initially employed for rooting. A maximum rate of root elongation was achieved in plants which had previously received a combination of NAA+IBA ($1+1 \text{ mg l}^{-1}$), (Fig. 3.2.1.4). The rate of elongation was suppressed at higher levels of NAA alone (Fig. 3.2.1.4). A concentration of 2 mg l^{-1} of IBA was also inhibitory to the growth rate of roots at all NAA levels tested (Fig. 3.2.1.4). Not all the primordia induced by the auxin treatments, which included a high level of BAP (0.5 mg l^{-1}), developed into adventitious roots. A mean maximum of 3 roots per root system was obtained on a combination of NAA+IBA at a concentration of $1+2 \text{ mg l}^{-1}$ respectively. However, in most cases, one of the induced primary roots became dominant.

iii. Branching of induced roots

In general, induced roots grown on an agar-medium were thick and unbranched (Fig. 3.2.1.5,A). Root branching was not observed during the hardening off period (1 month) (Fig. 3.2.1.5,B and Table 3.2.1.1). However, both dichotomously branching roots and short lateral roots started growing after the regenerated plantlets had been potted into a sand:peat mixture and maintained under greenhouse conditions for at least another month. Then, 37% of the plantlets appeared to be mycorrhizal (Fig. 3.2.1.5,C & D and Table 3.2.1.1). Maximum infection (72% mycorrhizal plantlets) was achieved after another 4 months in the greenhouse (Fig. 3.2.1.5,E & F and Table 3.2.1.1). The proportion of

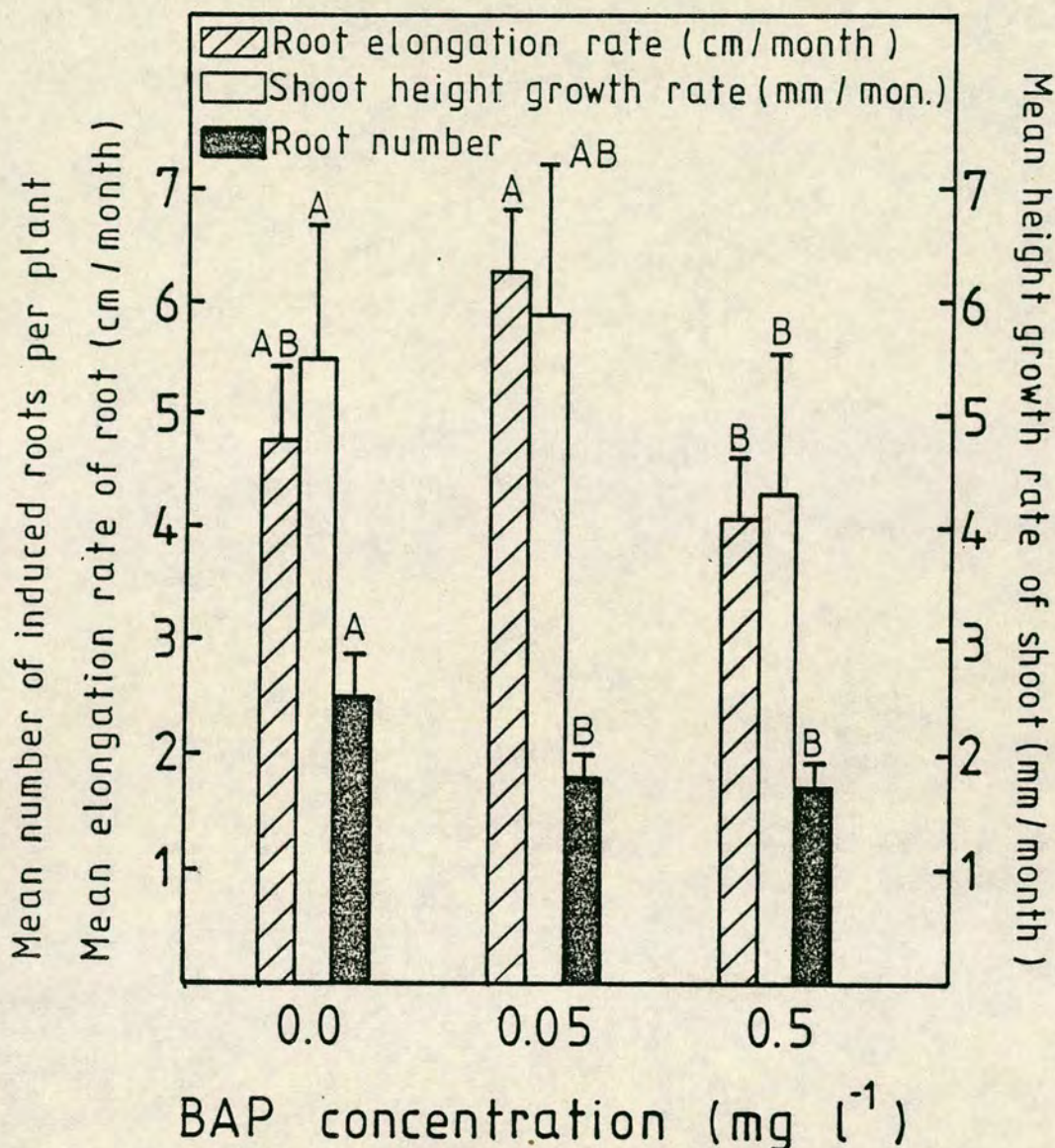


Fig. 3.2.1.3 The effect of benzylaminopurine (BAP), initially combined with auxins for root induction on axillary-derived shoots of *Pinus brutia*, on subsequent root and shoot characteristics of the plantlets 6 months after transplanting from agar into soil. 72 plantlets were used for each combination. Different letters above any two bars indicate that these two means are statistically different at $P=0.05$ level in \square and \blacksquare , at the $P=0.01$ level in ▨ (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

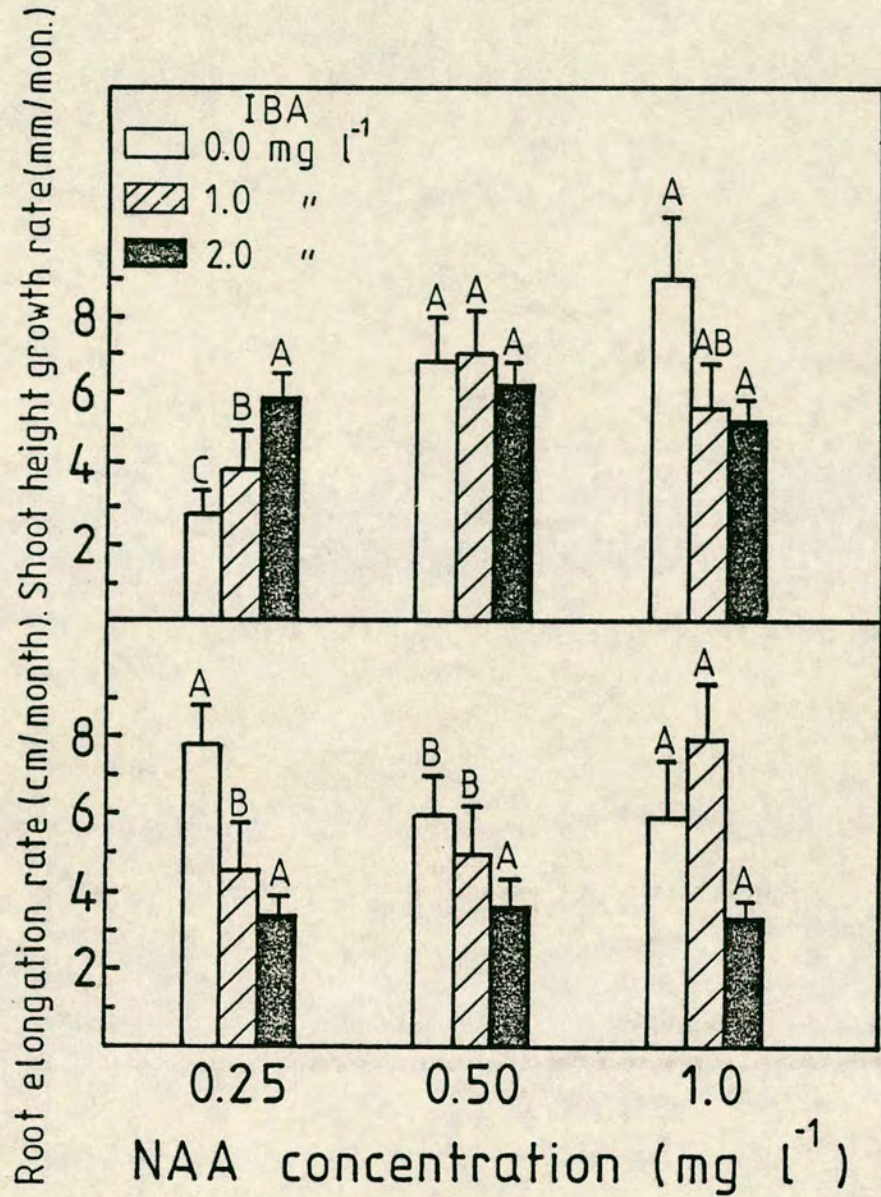


Fig. 3.2.1.4 The interaction of NAA and IBA (all with 0.05 mg l⁻¹ BAP), initially applied for root initiation on axillary shoots of *Pinus brutia*, on subsequent root elongation and the corresponding shoot height growth rate of regenerated plantlets 6 months after transplanting from agar into soil. 12 plantlets were used for each combination. Different letters above any two bars indicate that these two means are statistically different at P= 0.01 level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

Table 3.2.1.1 Effect of the age of plantlets of *Pinus brutia* on the production of mycorrhizal branches.

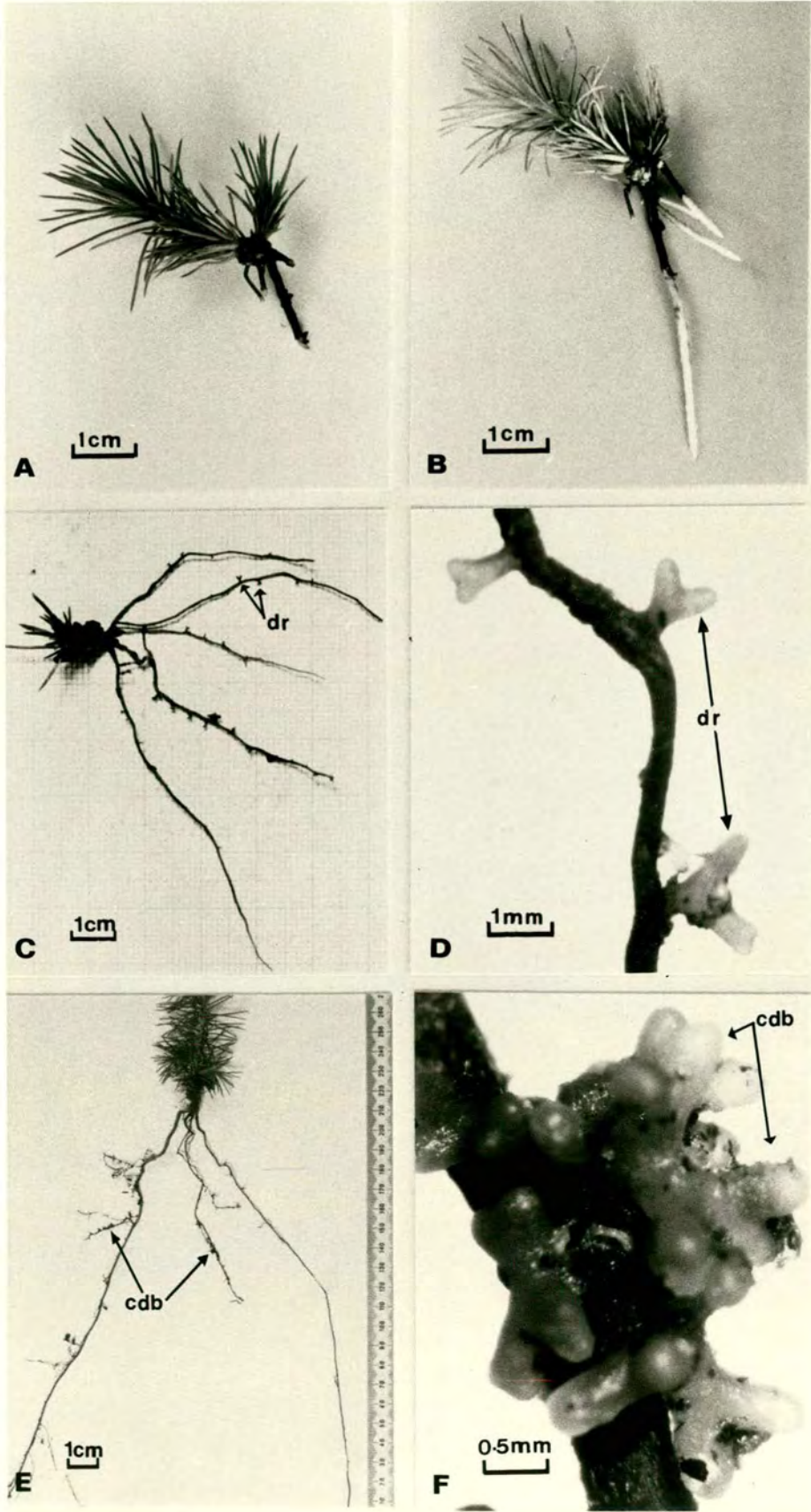
Time (month)	Total No. of plantlets	No. of plantlets with mycorrhizal branches	% of plantlets with mycorrhizal branches
1	60	0	0
2	60	22	37
6	60	43	72

Table 3.2.1.2 Characteristics of induced roots in axillary-derived shoots of *Pinus brutia* Ten.. 12 days after incubation on half-strength modified Schenk and Hildebrandt (SH) medium (Abdullah et al. (1985) supplied with growth substances, and 10 weeks on various root induction media.

Root-induction media	% of rooted shoots	Mean* no. of roots per shoot	Mean* length (cm) of induced root	Associated callus growth
Mod. SH medium	75 \pm 4.4a	3.1 \pm 0.1a	6.6 \pm 0.8a	(+)
Tap water	26 \pm 4.7b	1.3 \pm 0.2b	5.2 \pm 1.0a	(-)
Aerated-tap water	64 \pm 5.5a	2.8 \pm 0.2a	5.8 \pm 0.7a	(-)
CV (%)	33	33	44	

* Means in columns not followed by a common letter are statistically different at $P=0.001$ level (Duncan's multiple-range test). CV, coefficient of variation. (+), excess callus growth, (-), little callus growth.

Fig. 3.2.1.5, A-F Formation and development of adventitious roots and subsequent formation of mycorrhizal roots on *Pinus brutia* shoots grown from axillary buds on seedling plant material. Fig. A. Adventitious root formation on an induced shoot. Fig. B. The same rooted shoot one month after transplanting from agar into soil (under mist irrigation). Note the unbranched roots. Fig.C. The development of dichotomous secondary roots on induced primary roots. Fig.D. The secondary roots with a single dichotomy. Fig. E. Root system of plantlet, 6 months after transplanting from agar into soil, exhibiting mycorrhizal branching of varying lengths. Fig. F. The apex of secondary root proliferating into a cluster of numerous short dichotomous branches.



each individual root system infected was generally high, but was reduced on those root systems that had been induced on high IBA (2 mg l^{-1}) or high BAP (0.5 mg l^{-1}) (Fig. 3.2.1.6).

iv. Rate of shoot height growth (mm/month)

Six months after transplanting from sterile agar into soil, the higher growth rate of the shoot occurred on the root systems that had been initially induced on low or zero BAP (Fig. 3.2.1.3). There were also residual effects on shoot growth of IBA and NAA: where the maximum elongation rates were achieved when the NAA was high and IBA was absent (Fig. 3.2.1.4). Considering all the data together, extension growth was strongly correlated with the extent to which the roots were mycorrhizal (Fig. 3.2.1.7).

v. Survival of established plantlets

By following the optimal sequence of hardening off described in chapter 2, a high survival (89-100%) of plantlets was achieved. Most of the mortality occurred with plantlets that had initially been rooted on medium with high levels of IBA (2 mg l^{-1}) or BAP (0.5 mg l^{-1}).

B. Influence of different root induction media

i. Quantitative and qualitative characteristics of induced roots

Aeration of the tap water employed affected the rate of rooting, the number of roots formed and their final length (Fig. 3.2.1.8 and Table 3.2.1.2). The formation of roots on treated shoots maintained on a sterile-agar medium occurred 2 weeks earlier than shoots rooted in aerated-tap water. However, rooting in agar decreased with time so that after eight weeks there was little difference between agar and aerated water (Fig. 3.2.1.8).

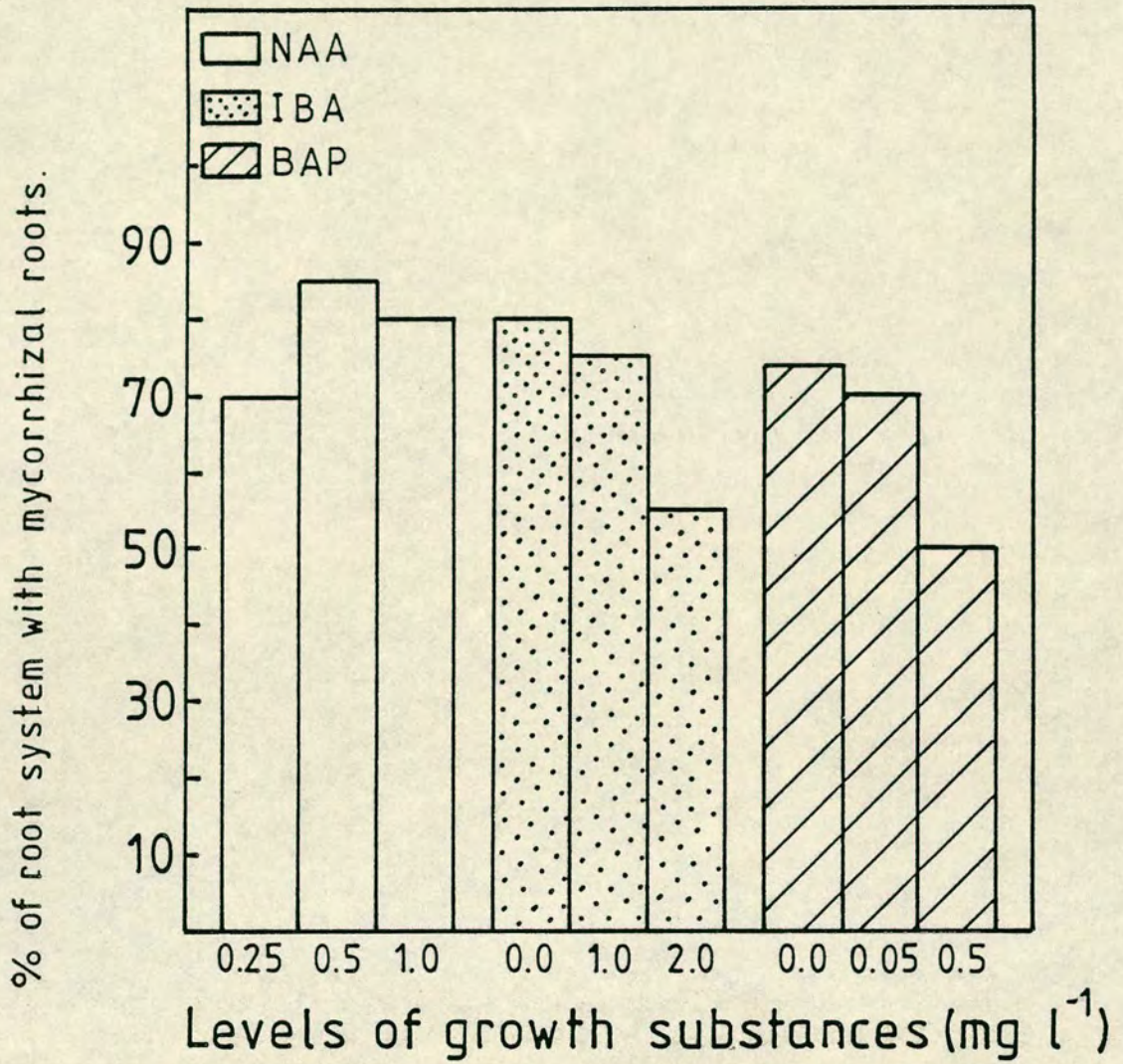


Fig. 3.2.1.6 The effect of various growth substances, initially employed for root induction on axillary shoots of *Pinus brutia*, on subsequent formation of mycorrhizal roots in regenerated plantlets 6 months after transfer from agar into soil. Observations were from 27-63 plantlets for each treatment.

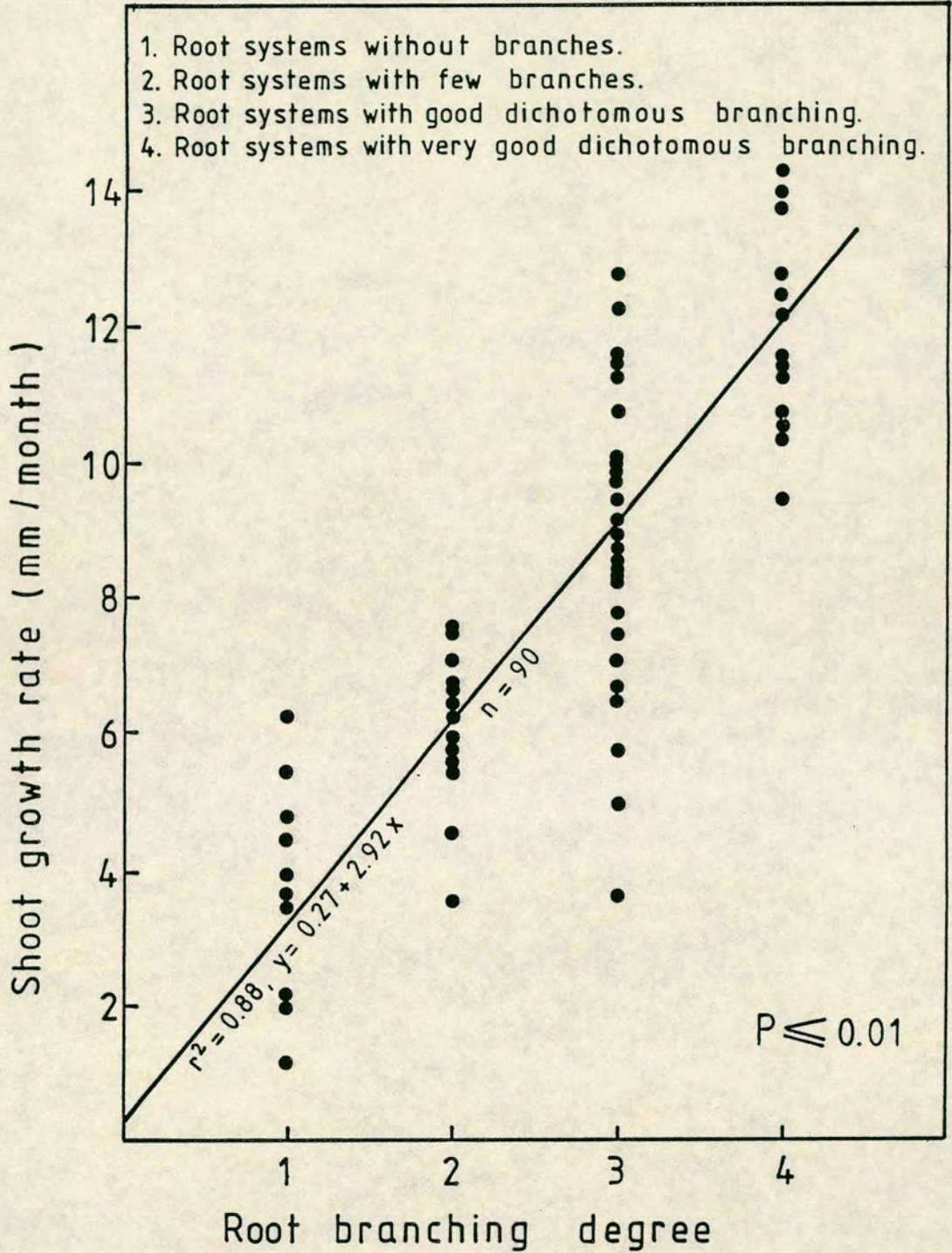


Fig. 3.2.1.7 Growth rate of shoots of *Pinus brutia* in relation to the rank score of mycorrhizal formation 6 months after transplanting from agar into soil.

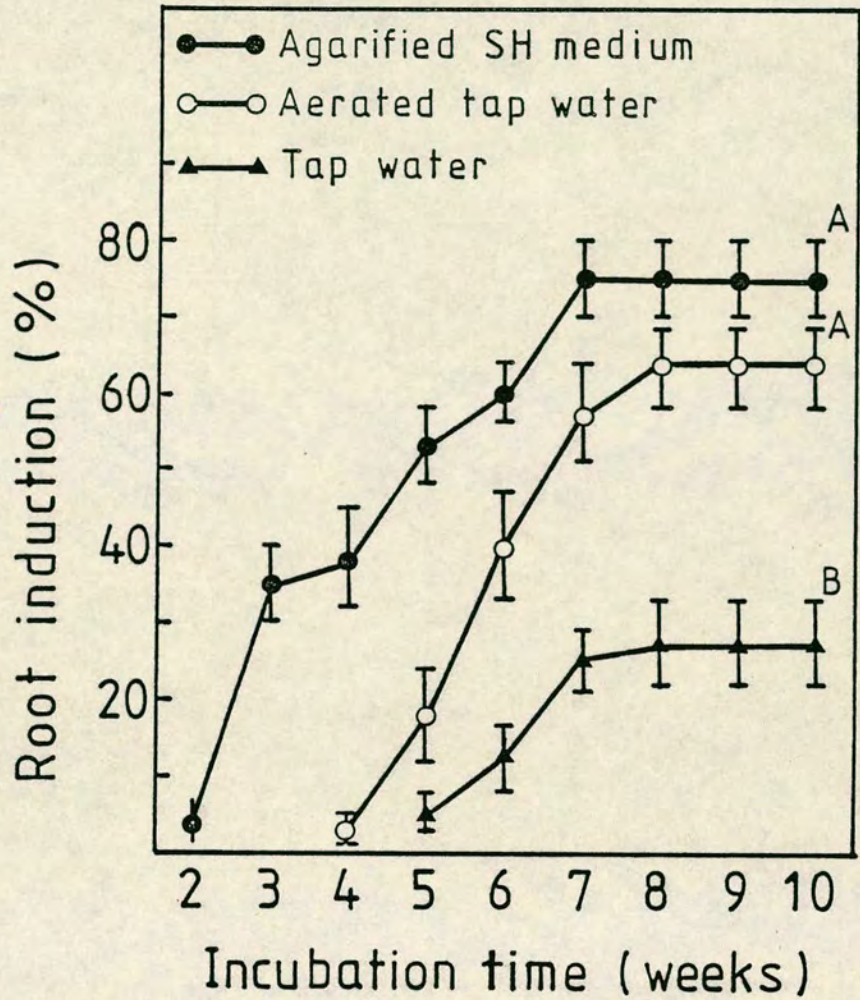


Fig. 3.2.1.8 Effect of three rooting media on the frequency of root-formation on axillary-derived shoots of *Pinus brutia*. 12 days after treatment on half-strength Schenk and Hildebrandt (SH) medium supplied with growth substances, and another 10 weeks on the above rooting media. 45 shoots were used for each treatment. Different letters above any two bars indicate that these two means are statistically different at $P=0.001$ level (Duncan's multiple-range test). Vertical bars represent the standard error of the mean.

ii. Callus growth

A reduction in the growth of callus at the bases of the treated shoots rooted in water was observed (Table 3.2.1.2), but unaerated cultures sometimes developed fungal infections associated with the callus.

iii. Survival of rooted shoots

After hardening off and establishment into soil in the greenhouse, the plantlets which had been rooted in aerated-tap water survived better than those rooted on a sterile-agar medium (98% versus 89% respectively).

II. Adventitious root formation on adventive shoots grown from seedling-needles

After 10 weeks on the induction medium, 64% (out of 45 shoots) of treated shoots had produced adventitious roots. Subsequently, 86% of the rooted shoots survived transplanting into soil. Following the sequence of shoot production, multiplication, and root induction, an average of 32 plantlets per seedling were obtained within 26 weeks.

III. Adventitious root formation on shoots induced on mature fascicle needles

Roots were visible only on those shoots treated with growth substances for 12 days. After 8-10 weeks on the induction medium, only 16% (out of 45 shoots) of the treated shoots rooted. Following the sequence of hardening off and establishment described in chapter 2, all of the rooted shoots were capable of surviving the transfer into soil.

3.2.2 ESTABLISHMENT OF PLANTLETS

A final assessment of the effectiveness of any *in vitro* micropropagation technique must be reserved until information becomes available on the performance *in vivo* of the plants produced. Accordingly, investigations were directed to obtaining the patterns of growth and development of adventive and axillary plantlets, and comparing them with seedlings of *Pinus brutia* one year after transfer into soil.

A. Growth characteristics of the plants

After a year in soil, it was apparent that seedlings had attained higher rates of growth in height and diameter, and accumulated more dry weight in their shoots than either type of *in vitro* plantlet. Comparing the two types of plantlets, shoot growth in axillary plantlets exceeded that of adventive plantlets (Table 3.2.2.1 and Fig. 3.2.2.1), but, adventive plantlets produced more frequent and longer tap roots than the seedlings. The root characteristics of the axillary plantlets were not very different from those of the seedlings (Table 3.2.2.1). It was also apparent that the developed root system was more fibrous in the seedlings than in either type of plantlet (Fig. 3.2.2.2). In general axillary plantlets produced the same total dry weight per plant as the seedlings (Table 3.2.2.1, Fig. 3.2.2.1 and 3.2.2.2).

There was also a considerable difference between the plantlets and the seedlings in the distribution of biomass (Table 3.2.2.1). The root-to-shoot dry weight ratio of the axillary plantlets converged

Table 3.2.2.1 Characteristics of shoot and root growth of three types of plant of *Pinus brutia* after a year in soil. Observations were taken from 30 plants of each.

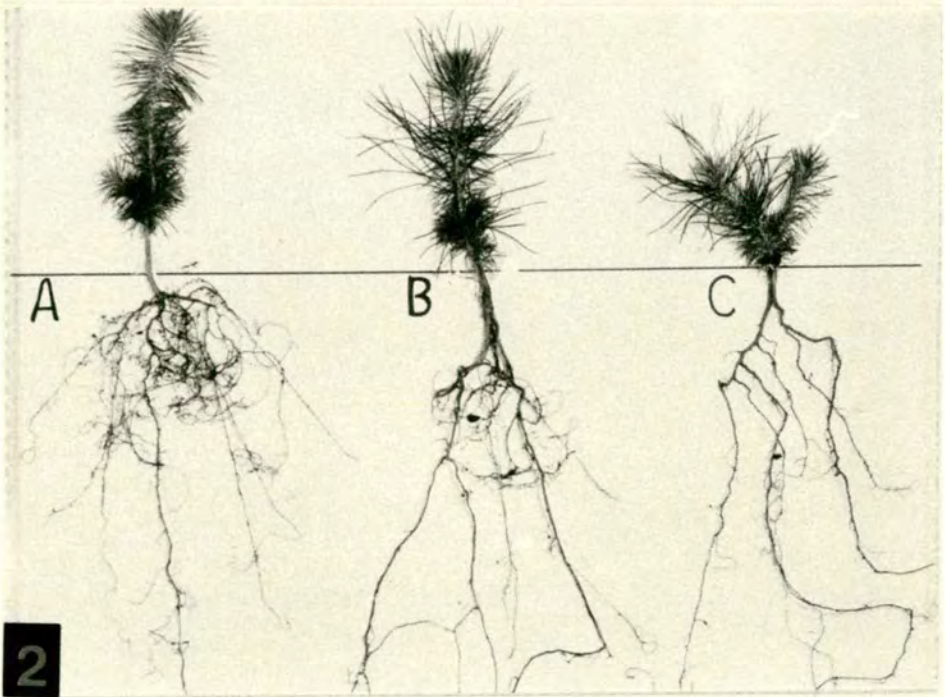
Characteristics growth	CV%	Type of Plant		
		Seedling	Axillary plantlet	Adventive plantlet
Shoot				
Height (cm)	18	14.7±0.3a	12.3±0.3b	8.5±0.4c ***
Diameter (mm)	21	2.8±0.1a	2.7±0.1a	2.1±0.1b **
Dry weight (g)	33	2.0±0.1a	1.6±0.1b	1.1±0.1c ***
Root				
Length (cm)	39	107±5.1a	110±8.0a	148±9.0c **
Diameter. (mm)	21	2.8±0.1a	2.4±0.1a	1.7±0.1b ***
Number	45	1.0±0.1a	1.7±0.1b	1.4±0.1b ***
Dry weight (g)	33	1.5±0.1a	1.4±0.1a	1.0±0.1b ***
Total dry weight (g)	30	3.5±0.2a	3.0±0.2a	2.1±0.1b ***

** Means in rows not followed by a common letter are statistically different at $P=0.01$ level, *** at $P=0.001$ level (Duncan's multiple-range test). CV, Coefficient of variations.

Table 3.2.2.2 The time of appearance of fascicle needles on the three types of plant of *Pinus brutia* within a year performance *in vivo*. Observations were taken from 30 plants of each.

Time (months)	% of plants forming fascicle needles		
	Seedling	Axillary plantlets	Adventive plantlets
5	0	0	0
6	0	0	0
7	0	0	13
8	0	0	30
9	0	0	30
10	3	13	30
11	3	30	36
12	30	43	50

Fig. 3.2.2.1-3.2.2.2 Patterns of growth and development of tissue culture-plantlets and seedlings of *Pinus brutia* after a year of performance in vivo. Fig. 1. typical 1 year-growth form of three types of plant. A, seedling; B, axillary plantlet; and C, adventive plantlet. Fig. 2. The same plants with their root systems.



towards that of the seedlings as the total biomass increased. In contrast, the comparison between these two types of plants on the one hand and the adventive plantlets on the other hand, showed that the corresponding ratio diverged as plant size increased (Fig. 3.2.2.3). The slope of the regression line in this case differed significantly from that of the axillary and seedling materials ($P = 0.05$).

B. Growth characteristics of fascicles

Fascicles started growing on the adventive plantlets four months earlier than on the other groups of plant. Appearance of the first fascicles on axillary plantlets coincided with that on the seedlings, but at a lower rate on the latter (Table 3.2.2.2). One year after transfer into soil, about 50% of both types of plantlets had fascicle needles but only 30% of the seedlings (Table 3.2.2.2). An investigation into the other characteristics of growing fascicles of the three types of plant showed no real differences. The same rate of accumulated dry weight in fascicle needles was displayed by the three groups of plants, as the specific leaf area and dry weight ratio (SLA) was about equal for all of them (Fig. 3.2.2.4). It was also evident that the influence of plant type on stomatal resistance, if any, was small. (Fig. 3.2.2.4).

C. Growth patterns and form of plants

A year after transfer into soil, adventive plantlets displayed four different patterns of growth form. More than half of the plantlets was either bushy or plagiotropic in growth, and only 16% displayed good straight form, whereas 31% were upright in form but bushy at the base

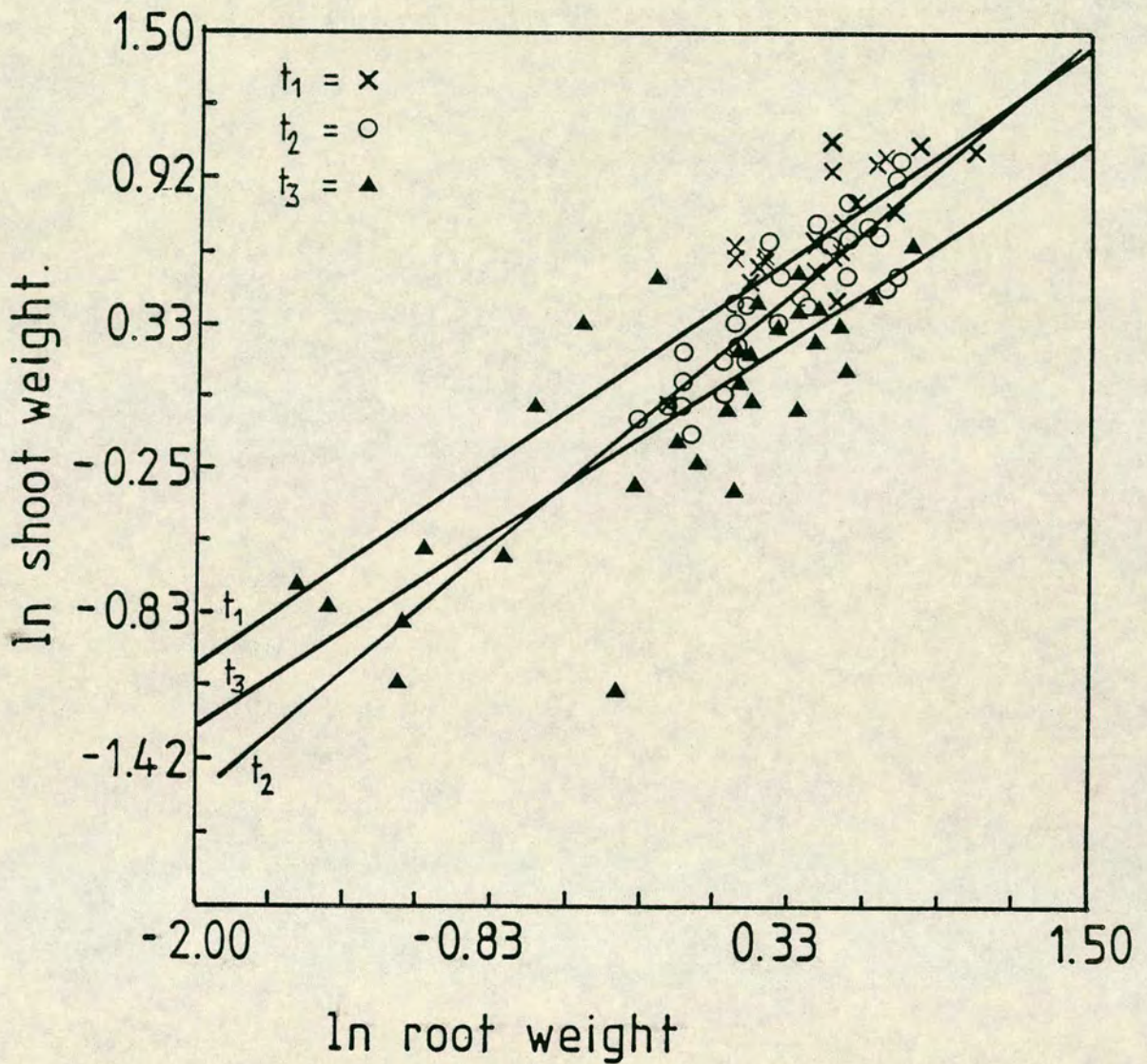
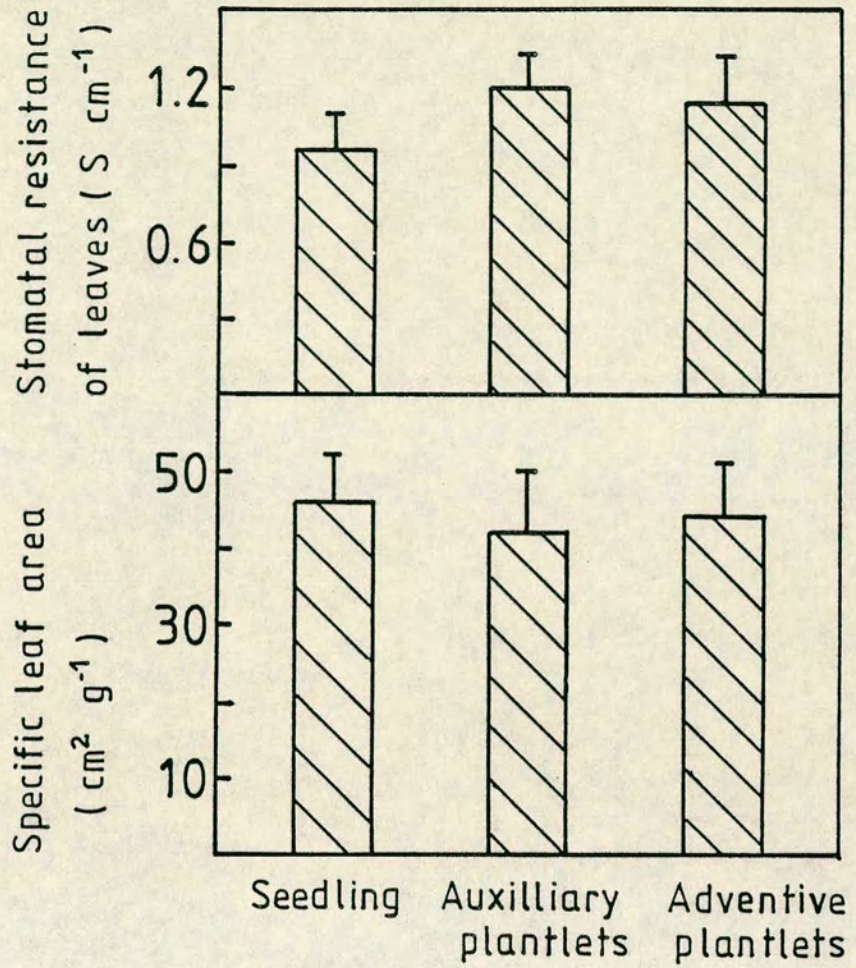


Fig. 3.2.2.3 Relationship between root dry weight and shoot dry weight for adventive plantlets(\blacktriangle), axillary plantlets (\circ), and seedlings (\times) of *Pinus brutia* after one year *in vivo*. The slope of the regression and the standard error are as follows: adventive, 0.621 ± 0.08 ; axillary, 0.872 ± 0.09 ; seedling, 0.733 ± 0.11 .



Types of plant

Fig. 3.2.2.4 Specific leaf area and dry weight ratio (cm² g⁻¹) and stomatal resistance of leaves to water vapor (s cm⁻¹) of three types of plant of *Pinus brutia* after one year of performance *in vivo*. Vertical bars represent the standard error of the mean.

(Fig. 3.2.2.5). On the other hand, 70% of the axillary plantlets resembled the seedlings in producing vigorous upright shoots, and the rest was of similar quality but bushy at the base. However the majority of the seedlings displayed a good straight form. (Fig. 3.2.2.5).

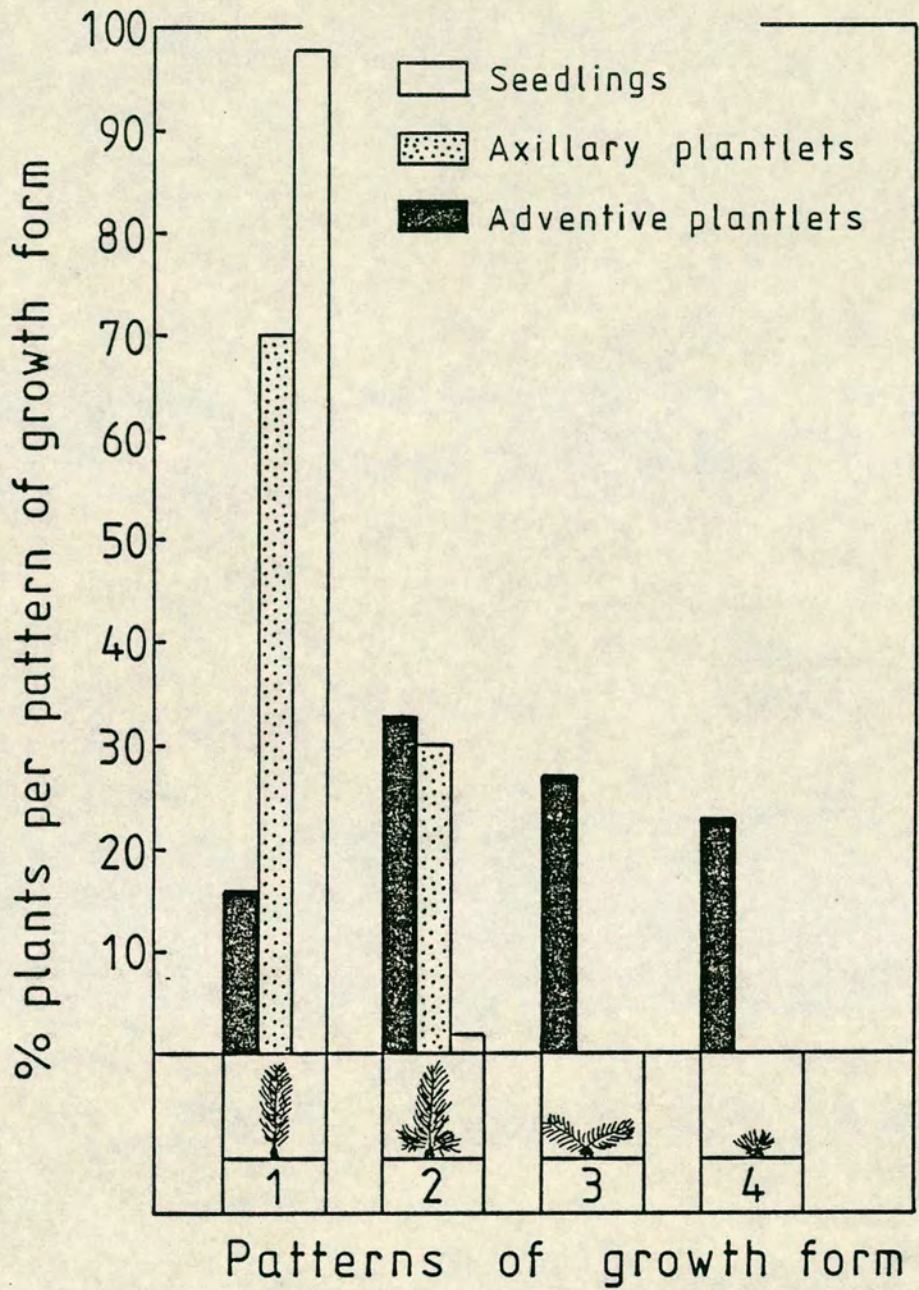


Fig. 3.2.2.5 The growth patterns of the three types of plant of *Pinus brutia* within a year of performance *in vivo*. 1, upright growth form; 2, upright growth form and bushy at the base; 3, plagiotropic growth form; and 4, bushy growth form.

Chapter 4

Discussion and Conclusions

The experimental data presented in this thesis point to the involvement of a multiplicity of influences at each stage of regeneration of *Pinus brutia* plantlets. Therefore, In an attempt to elucidate this complexity, the regenerative process is looked at from different perspectives, paying particular attention to the factors involved.

4.1 NUTRIENT MEDIUM

Certainly, for the successful regeneration of plants from culture, the nutrient medium must be suitable in chemical composition to meet the heterotrophic demands of the cultures. Results obtained in this laboratory reveal that organogenesis on Calabrian pine embryonic explants can be influenced by the mineral salt levels in the medium (Abdullah et al. 1985). Elsewhere, this has been confirmed for other species (Horgan and Aitken 1981, Bornman 1983, Thompson and Zaerr 1982, Rumary and Thorpe 1984). Consistent with results obtained with *Pinus brutia*, SH medium has been found to be very useful by other workers (Reilly and Brown 1976, Reilly and Washer 1977, Horgan and Aitken 1981, Jansson and Bornman 1983, Rumary and Thorpe 1984). Perhaps significantly, SH medium is characterized by a high concentration (24.7 mM) of potassium, whilst the concentration of calcium (1.6 mM) is intermediate between that in LS medium and GD medium. In fact, the beneficial effect of potassium on organogenesis has been reported (David et al. 1978, David 1982), and perhaps this effect can be linked with the high concentration of this cation that is often found in the meristematic regions of plants (Devlin 1975). The level of calcium, and its ratio to other macroelements, is also considered to be important (Bornman 1983, David et al. 1984). These observations are

not surprising in view of the accepted role of Ca^{++} in sustaining meristematic activity of the shoot apex (Bornman 1983). and its direct role in cell wall formation. Moreover, the vitamins present in the SH medium are richer in concentration and variety, particularly the level of myo-Inositol. Although the role of this vitamin is not completely understood, it is involved in the synthesis of phospholipids and in cell wall pectins (Anderson and Wolter 1966), and in promoting bud formation in cultures of *Ulmus campestris* (Jacquiot 1966). The phenomenon of yellow shoot production in tissues cultured on SH medium (Horgan and Aitken 1981, Abdullah et al. 1985) is likely to be related to the low iron concentration. In this laboratory this problem has been overcome by an increase in the iron content of SH medium relative to the level used in LS medium. On the other hand, LS medium, which is similar to MS medium, is characterized by a high level of ammonium. Indeed, there is evidence that high levels of this cation can reduce cell growth in some plant systems (Gamborg and Shyluk 1970), and that it reduces the survival rates and development of needle primordia on cultures of Douglas fir (Thompson and Zaerr 1982) and *Pinus williciana* (Konar and Singh (1980). This may explain why the dilution of MS medium has recently gained widespread use for the regeneration of some conifers (Cheng 1979, Jelaska et al. 1982). Clearly it is vital to establish a medium that is compatible with the cultured species, and it would seem that the lack of systematic studies to this end might explain why to date only a few conifers have been regenerated *in vitro*.

To support the further development and growth of adventitious and axillary buds induced on cultured seedling-needles, shoot explants, and excised fascicles of *Pinus brutia*, the SH initiation medium had to be

diluted to half strength and the sucrose content reduced to 2%. Similar observation has been reported with many other species (e.g. *Pinus radiata* (Reilly and Brown 1976), *Pinus taeda* (Mehra-Palta et al. 1978), *Picea abies* (Jansson and Bornman 1980), *Pinus contorta* (Von Arnold and Eriksson 1981). Recent studies support the hypothesis that initiation of organized development *in vitro* involves a shift in metabolism (Obata-Sasaemoto et al 1984). This is likely to be a high energy requiring process in which a significant accumulation of starch precedes the initiation of meristemoids and primordia formation, as has been observed in tobacco culture (Thorpe and Murashige 1968). Also a rapid depletion of lipids and free sugars by 10-fold and 6-fold respectively was evident during bud induction in the culture of *Pinus radiata* (Biondi and Thorpe 1982). However, the lower nutritional requirement for bud development and elongation in the succeeding stages might be due to utilization of some of the energy reserves during the first stage, although it is probable that inhibition of this process at a high level of carbohydrates is partly osmotic (Thorpe 1982).

In contrast to the initiation of buds, the subsequent induction of adventitious roots was achieved on a medium containing half-strength mineral salts but with an even lower sucrose level of 0.8%. It is apparent that lowering the mineral and sugar concentration in the applied basal medium usually favours rooting in gymnosperms (e.g. *Thuja plicata* (Coleman and Thorpe 1977), *Pinus taeda* (Mott and Amerson 1981), *Pinus monticola* (Amerson and Mott 1982), *Picea mariana* and *Picea glauca* (Rumary and Thorpe 1984), *Pinus contorta* (Patel and Thorpe 1984). Perhaps these results merely point to the need for some kind of deficiency in the standard nutrient culture when it is required to

induce rooting, and one good piece of support for this hypothesis is that root initiation was accomplished on *Pinus brutia* shoots using tap water as the root induction medium. The tap water of Edinburgh is notably deficient in dissolved solids and it is possible that the induction of the adventitious roots is triggered by a nutrient-deficient environment. Recently, high rooting of >80% in *Picea mariana* & *Picea glauca* (Rumary and Thorpe 1984), and 75% in *Pinus sylvestris* (Gronroos and Von Arnold 1985) has been obtained by the addition of activated charcoal in the rooting medium. The effect of AC may be, at least in part, the result of adsorption of ions on the active particles with subsequent nutrient unavailability (Weatherhead et al. 1979). Alternative approaches for rooting of conifers under non-sterile conditions using substrates other than agar have been successfully established for *Pinus taeda* (Mehra-Palta et al. 1978) and *Pinus radiata* (Horgan and Aitken 1981). Other attempts were only partially successful (Cheng 1976, Chalupa 1977, David et al. 1982). The procedure described in this investigation, however, with rooting of 64% in tap water offers a simple and reliable method for induction of roots. Of equal importance is that by applying this procedure a reduction in the growth of callus at the base of the rooted shoots of Calabrian pine was obtained. Indeed, this technique has been attempted with conifers *in vitro* to suppress callus growth relative to growth of the roots (Sommer and Caldas 1981). It is worth noting that after the hardening off and establishment into soil in the greenhouse, the propagules that rooted in the aerated tap water survived better than those rooted on the sterile-agar medium. This was also observed in *Pinus taeda* (Mehra-palta et al. 1978) and *Pinus radiata* (Horgan and

Aitken 1981), where roots that formed directly in soil survived better than those formed in agar.

The data available on nutritional requirements are tentative and not sufficient to make an overall critical evaluation of the role of mineral ions in the control of plant development. However, they may point to a critical concentration of nutrients which induce differentiation. As yet, there is no universally applicable formula for a nutrient medium, although limited guiding principles on the role of some nutrient elements may be, and have been, enunciated. The choice of nutrient medium is likely therefore, until the near future, to be one of the more empirical aspects of the culture of gymnosperms *in vitro*. It is certain now that there is a gradual decrease in the nutritional and sugar requirement for cultures throughout their regeneration, and this must reflect a tendency of the culture to become gradually less heterotrophic. This diversity in nutritional requirements points to the existence of a sequence of physiological events in the regeneration process rather than one event.

4.2 PHYSICAL CULTURE ENVIRONMENTS

(i) Physical properties of the nutrient medium

Results of the present study show that the cultured needles were regenerated more actively on the agar medium than on the liquid medium, at all levels of cytokinins. This has also been reported with *Pinus radiata* (Reilly and Brown 1976), *Picea mariana* and *Picea glauca* (Rumary and Thorpe 1984). It is possible that the initial uptake of constituents by the tissue in the liquid medium may be much faster than from agar, since in the former case a better contact with the medium is afforded. This effect has been reported even when the agar

concentration is reduced as in the culture of Norway spruce (Von Arnold and Eriksson 1979_b). Nevertheless, occasionally the development of induced buds of Norway spruce stopped on a medium containing only 0.3% agar (Von Arnold and Eriksson 1979_b); also explants of *Pinus brutia* left in liquid medium for a period longer than 3-4 weeks turned brown, and using liquid rather than agar medium reduced shoot formation on cultured explants of *Picea mariana* and *Picea glauca* (Rumary and Thorpe 1984). It is possible that some essential nutrients may leak from the cultured explants into the liquid medium. Also the use of liquid medium may limit the availability of oxygen for continuous growth of cultured tissues. Data presented on rooting of Calabrian pine shoots in water reveal that aeration of this medium is critical to the quantity and quality of the induced roots. In this connection, it is clear that bubbling the air seems to be more efficient and economic for aerating the water-medium than using bulky shakers.

A real difficulty with many tissues cultured in liquid media is the occurrence of 'vitrification' (Yeoman 1986). In fact, the further development and survival of the vitrified organs induced on the cultured needles in liquid has not been followed in the present study, but it has been reported that vitrified shoots produced on *Pinus radiata* explants seemed to be unhealthy and difficult to root and unable to survive the transfer from sterile conditions to soil (Horgan and Aitken 1981). Apart from whether ethylene is involved (Kevers et al. 1984), or cytokinins (Debergh 1983), it is now clear that lowering the concentration of agar causes an increased water potential of the cultures which results in the vitrification of the tissues (Debergh 1983, Von Arnold and Eriksson 1984). Occasionally, vitrification of

embryonic culture of *Pinus brutia* has been observed after several subcultures (the sixth harvest of shoots) (Abdullah et al. 1985). This might be related to changes in culture conditions due to inadvertent alteration in pH or temperature, but, why vitrification occurs in one laboratory and not in another (Debergh 1983) is still difficult to answer. Even if vitrification is reversible as in *Pinus radiata*, the time required is rather long (8-10 months) (Horgan and Aitken 1981). Even so, the intensive care suggested by Von Arnold Eriksson (1984) to ensure acclimatization and survival of vitrified shoots and plantlets of *Picea abies* after outplanting to soil seems economically unreliable. Vitrification is still poorly understood and has not as yet been investigated thoroughly. Certainly with the need to simplify handling procedures which means the increased use of liquid media for mass propagation, i.e. somatic embryogenesis of forest species, the associated problems with using a liquid media must be solved in the near future (Yeoman 1986). Also it seems evident that using liquid medium, aeration and availability of nutrients are important limiting factors for the growth of plant tissue.

It is possible to conclude that agar is best but that its concentration should be as low as possible to ensure the availability of nutrients and hormones but without involving vitrification. On the other hand, since crude agar contains a variety of substances as impurities which may affect the growth and differentiation of cultures, purified agar should be used whenever possible, especially for experimental work. Satisfactory results have been achieved in our laboratory using Difco 'Bacto' agar for most of the cultures.

It is evident from results obtained on the rooting of Calabrian pine

shoots on different root induction media that agar media support early formation of adventitious roots, but in long term incubations of 8 weeks, rooting slows down in the agar, whereas it continues in aerated water eventually equalling that on the agar medium. This reduction in growth in the agar presumably results from the relatively large resistance to diffusion in the solid medium. Transfer of CO₂ and any waste metabolites away from the root, and transfer of oxygen to the root, may limit root development. Also the agar medium may mechanically inhibit root hair formation. So far no-one has obtained functional roots in agar. The influence exerted by the agar here seems to be physical rather than chemical

(ii) Light

Morphogenesis *in vitro* is influenced by the diurnal light/dark cycle, and it seems that a relationship exists between the photoperiodic demand of the intact plants and the culture (Murashige 1974). Investigations on embryonic tissue of *Pinus brutia in vitro* have clearly demonstrated that a 16 h photoperiod elicits a higher yield of adventitious buds than continuous illumination (Abdullah et al. 1985). Subsequently, this photoperiodic regime has been employed successfully with explants from all of the sources used in present study, and for all stages of regeneration. These observations are in agreement with those found in *Pinus contorta* (Patel and Thorpe 1984), *Picea abies* (Von Arnold 1982_b), and in *Pinus rigida* (Patel et al. 1986). Although, most of the other studies on cultures of conifers have not critically evaluated the daily light requirement, of practical interest is the observation that a photoperiodic regime (mainly 16 h per day) has been reported to be satisfactory for many of these

cultures, including the majority of pine species which have been regenerated *in vitro* (Bonga and McInnis 1975, Brown and Sommer 1977, Bonga 1977, Winton and Verhagen 1977, Mehra- Palta et al. 1978, Bornman and Jansson 1980, Bonga 1981, Aitken et al. 1981, Jelaska et al. 1982, David et al. 1982, Rancillac et al. 1982, Patel and Thorpe 1984, Rumary and Thorpe 1984). The light intensity required for bud initiation in many conifers seems to be in the range of ca. $80 \mu\text{mol m}^{-2} \text{s}^{-1}$ (e.g. *Pinus resinosa* (Bonga and McInnis 1975), many conifers (Brown and Sommer 1977), *Pinus radiata* (Reilly and Washer 1977), *Pinus monticola* (Mott and Amerson 1981_b), *Pinus pinaster* (David et al. 1982), *Pinus rigida* (Patel et al. 1986). This has been confirmed in this study for Calabrian pine. In fact, photosynthesis is not a necessary activity, except perhaps during the latter part of the root induction and plantlet development stage (Murashige 1974), since external supply of sucrose is provided. Light is therefore most important in plant tissue culture for its effect on photomorphogenesis (Murashige 1974, Hughes 1981, Dougals et al. 1982, Villalabos et al. 1984_a). Also of interest to note, is that a lower intensity ($70 \mu\text{mol m}^{-2} \text{s}^{-1}$) is satisfactory for rooting of *Pinus brutia* shoots *in vitro*. The similar observations have been reported by others (Coleman and Thorpe 1977, Mott and Amerson 1981_a, Rancillac et al. 1982, Rumary and Thorpe 1984, Patel and Thorpe 1984). Presumably, a low light intensity is preferable at this stage to avoid an inhibition of rooting perhaps because of the destruction of a major part of IAA by excess blue light (Yamakawa et al. 1979). Perhaps a higher light intensity becomes necessary later, prior to transfer of rooted shoots into soil, in view of the increasing requirement for photosynthesis and the need to acclimatize the culture

prior to exposure to full daylight.

(iii) Temperature

It is advisable to adjust the culture temperature inside the containers used (flasks, Petri dishes.....etc.) rather than merely within the growth chamber. In this laboratory, a differences of 4°C has been found between the temperature of the growth chamber and inside the plates (using fine thermocouples inserted inside the Petri dish plates, Abdullah et al. 1985). This aspect has been neglected by most workers, and so it seems that the optimal temperatures in the tissues are higher than those reported. Identification of the exact optimal temperature for culture can be of advantage in increasing the rate of bud production, and to shorten the regeneration time in cultures of *Pinus contorta* (Patel and Thorpe 1984) and *Pinus rigida* (Patel et al. 1986). For the initiation stage, in *Pinus brutia* an average growth room temperature of $23 \pm 1^{\circ}\text{C}$ has been successfully used, which is equivalent to $26 \pm 1^{\circ}\text{C}$ inside the plate. Indeed, this is in the range of temperature (20-27° C) at which most culture tissues grow well (Murashige 1974). However, it seems evident that there is a general requirement for a lower temperature ($<25^{\circ}\text{C}$) when rooting most conifer shoots *in vitro* (Cheng 1977, Cheng and Voqoui 1977, Mott and Amerson 1981, Rancillac et al. 1982, Rumary and Thorpe 1984, Patel and Thorpe 1984, Patel et al. 1986). This holds true for rooting of *Pinus brutia*. Clearly, adequate carbohydrate reserves are essential to ensure good rooting of the shoots produced, and a high temperature above 25°C is likely to increase the depletion rate of these compounds.

It is certain that the identification of optimal incubation conditions can be of crucial importance in establishment of an

effective and reliable method of plant regeneration and establishment. This is equally true with regard to the future application of biotechnology to the propagation of forest trees, particularly to ensure efficient control of the environment on an industrial scale.

4.3 INFLUENCE OF GROWTH SUBSTANCES

(i) Induction and development of adventitious and axillary buds

It is generally accepted (e.g. Skoog 1971, Fosket 1980, Thorpe 1980) that the induction of organogenesis requires the application, via the culture medium, of an appropriate balance of the two primary morphogens auxin and cytokinin. However, the results presented here (see also Abdullah et al. 1985) reveal that cytokinin alone is sufficient to bring about shoot formation in cultures of *Pinus brutia*. This is also evident in a number of other conifers *in vitro* (e.g. *Picea abies* (Von Arnold and Eriksson 1979; Bornman 1983), *Pinus radiata* (Biondi and Thorpe 1982), *Pinus sylvestris* (Tranvan 1979; Bornman and Jansson 1980), *Pinus contorta* (Von Arnold and Eriksson 1981), *Pinus rigida* (Patel et al. 1986). However, the fact that auxin can be omitted in these cultures does not exclude the role of an auxin/cytokinin interaction in controlling the developmental process in these materials, as there may be involvement of endogenous auxins acting at organ-forming loci. The inclusion of auxin, in this study and that of Bornman (1983) and Rumary and Thorpe (1984) even at a low level of 0.01-0.05 mg l⁻¹ promoted unwanted callus growth not organogenesis. Measurement of cytokinin and auxin levels at organ-forming loci, is however technically impossible at present (Zaerr and Mapes 1982, Bornman 1985).

This study reveals that BAP is more potent, in terms of induction of adventitious buds, than the other cytokinins tested, and this holds true for cytokinin mixtures with BAP. This relative effectiveness of BAP has been reported in conifer culture systems by others (e.g. Webb

and Street 1977, Thomas et al. 1977, Cheng 1979, Bornman and Jansson 1980, Patel et al. 1986). but not by everyone (e.g. Reilly and Brown 1976, Von Arnold 1982_a, Mehra-Palta et al. 1978, Rumary and Thorpe 1984). This difference in requirement among conifers *in vitro* necessitates considerable experimental work, for individual species, to find the kind of growth substances required to supplement those already present in the explants.

Following the discovery of the diverse influences exerted by different cytokinins on developmental responses of conifers *in vitro*, came the suggestion that mixtures of cytokinins might increase the production of buds (Cheng 1975; 1979, Webb and Street 1977, Von Arnold 1982_a). Data to support this suggestion have recently been reported with *Picea glauca* and *Picea mariana* (Rumary and Thorpe 1984). This is also true for *Pinus brutia*, as an increased bud production per cultured needle was attained on a mixture of two cytokinins. BAP+Kin proved to be the best combination, and BAP+Kin rather than BAP alone was more effective in promoting axillary bud development, and subsequent multiplication of buds and shoots on shoot explants. Eventually BAP+Kin (each at 5 mg l⁻¹) was applied successfully for the activation of dormant meristems in fascicles of *Pinus brutia*. This was previously shown to be the case for buds *in vitro* from mature trees of *Picea abies* (Von Arnold 1984).

Several generalizations concerning the quantitative requirement of cytokinins for bud production emerged from the present investigation which seem to support the published data of others. The quantitative requirement mainly depends on the explant source, which presumably reflects the diversity of physiological state of the explants. Indeed,

in this species, there is a low requirement of cytokinin, in terms of both the quantity and the exposure time, for axillary bud development. This is evident in culture system used for axillary bud production in *Pinus nigra* (Jelaska et al. 1982). In contrast, David et al (1978) reported a higher concentration (10 mg l^{-1}) of exogenous BAP for *Pinus pinaster in vitro*. The developmental state of axillary buds in the material used in the present study could explain why such a low level of cytokinin was sufficient with *Pinus brutia*. Recent success in axillary bud production of *Pinus lambertiana* and *Pseudotsuga menziesii* was achieved even on a medium devoid of exogenous growth substances (Gupta and Durzan 1985), although, here large explants with a high level of endogenous hormones provides a possible explanation. On the other hand, *de novo* initiation of primordia on non-meristematic tissues of this species generally requires a higher concentration ($3-10 \text{ mg l}^{-1}$) of exogenous cytokinins. A relatively high level of cytokinin ($5-10 \text{ mg l}^{-1}$) is required for an optimum responses with the needle culture system used with this species and other conifers (Reilly and Brown 1976, Chalupa 1977, David et al. 1978, Jansson and Bornman 1980). This can be related to the xeromorphic nature of the conifer leaf (both the low ratio of leaf surface to volume and the heavy cuticular layer) that could impede the uptake of cytokinins. A lower requirement ($1-5 \text{ mg l}^{-1}$) is normal for embryos and cotyledons (e.g. Cheng 1975, Reilly and Washer 1977, Webb and Street 1977, Mehra-Palta et al. 1978, Tranvan 1979, Konar and Singh 1980, Minocha 1980, David et al. 1982, Abdullah et al. 1985, Patel et al. 1986).

Certainly, the availability of growth substances in cultured cells and tissues is mainly affected by the concentration and uptake of the

cytokinins in the culture medium (Jacobsen 1983). Short exposure of *Pinus brutia* needles to high levels of BAP do not cause a high morphogenic response. It is likely that the needle explants used here could impose severe limitations on cytokinin uptake. Recent study suggests a passive pattern of cytokinin uptake which may be restricted to microchannels in the cuticle. A significant diffusion barrier on cotyledons of *Picea abies* and *Pinus sylvestris*, pulse-treated with cytokinin, has been reported (Bornman 1983, Vogelmann et al. 1984, Staden et al. 1986).

(ii) Formation of shoots from adventitious and axillary buds

Results obtained from culture of various explants of *Pinus brutia* have revealed the necessity to transfer the induced adventive and axillary buds onto media without growth substances. The same observation has been reported by others (e.g. Cheng 1975; 1976, Chalupa 1977_b, Reilly and Washer 1977, Konar and Singh 1980, Aitken et al. 1981). This is in agreement with the findings of Mehra-Palta et al. (1978), Boulay (1979_b, Von Arnold and Eriksson (1981), Bornman (1983), Patel and Thorpe (1984), and Rumary and Thorpe (1984) the inclusion of activated charcoal in the elongation medium has proved to be of advantage for shoot elongation and development. Although, the role of charcoal is not completely understood, it is presumed to absorb excess growth substances, phenolics, and toxins formed during autoclaving and culture (Weatherhead 1978; 1979, Misson 1982). Activated charcoal, however, should be used with care as an inhibitory effect on the growth of dormant meristems of *Pinus brutia* fascicles has been shown in this study. Information on how the growth substances, which are initially required for the induction of buds, prevent the

formation of shoots is lacking. The inadvertent triggering of dormancy in buds already present as a result of aseptic culture has been put forward as an explanation (Mott 1981). Using high levels of cytokinin or auxin in the initiation stage might also impose residual effects on shoot formation. This is evident in cultures of many conifers e.g. *Pinus taeda* (Mehra-Palta et al. 1978, selected members of *Juniperus*; *Sequoia*; *Taxus*; *Thuja*; & *Tsuga* (Amos and McCown 1981), *Pinus brutia* (Abdullah et al. 1985), *Pinus rigida* (Patel et al. 1986). It is interesting that the elongation of induced buds on explants of *Pinus radiata* (Von Arnold 1982_a) and *Pseudotsuga menziesii* (Cheng 1979) was affected by cytokinins applied for the initiation stage. The ability of Kin to hasten the elongation of buds *in vitro* has been particularly related to its stimulatory effect in enhancing the formation of needle primordia.

(iii) Regeneration of plantlets

Formation of adventitious roots

Initiation of roots was found to be especially dependent upon auxin. This is in accord with the usual finding that this hormone is a requirement for adventitious root induction (Scott 1972, Haissig 1974, Fosket 1980). The present data also indicate that the potency of auxin is significantly influenced by its type and applied concentration. NAA is more potent than IBA in this respect. This finding is consistent with the fact that most gymnosperms, including all pine species so far regenerated *in vitro* have been rooted on this auxin (e.g. Thomas et al. 1977, Mehra-Palta et al. 1978, Cheng 1979, Aitken et al. 1981, Mott and Amerson 1981_a; 1981_b, Rancillac et al. 1982, Amerson and Mott 1982, Patel et al. 1986). Cultured shoots also responds according to the

auxin level employed. However, a mixture of two auxins elicits maximum rooting, a fact that has been reported by others (Aitken et al. 1981, Horgan and Aitken 1981, Patel et al. 1986).

Although cytokinins are known to inhibit rather than promote adventitious rooting (Scott 1972, Fosket 1980), the positive results achieved with a low level of BAP, confirm that this growth substance can act as a stimulator when it is applied at low concentration (Thomas et al. 1977, Mehra-Palta et al. 1978, Cheng 1979, Bornman and Jansson 1980, Mott and Amerson 1981_a; 1981_b, Amerson and Mott 1982). Indeed, cytokinins have been successfully used for *in vitro* rooting of pine species in the range of 0.01-0.5 mg l⁻¹ with maximum rooting of 75% achieved so far at CK:auxin ratio of 1:5 in *Pinus taeda* (Mott and Amerson 1981_a) and 82% rooting obtained at ratio of 1:1 in *Pseudotsuga menziesii* (Cheng 1979). However, in this system a lower ratio of 1:60 (CK:auxin) produces rooting of 73%. On the other hand, the high level of BAP applied in this study evokes a reduction in the frequency at which the shoots form roots, as well as decreasing the mean number of roots on the induced root system. It might be reasonably assumed from these results that the high concentration of BAP (0.5 mg l⁻¹) applied might block the action of the auxin employed in some process before and after the initiation of the primordia, and this could explain why not all the root primordia produced with this treatment continue to develop into roots.

The most commonly encountered difficulty in the micropropagation of mature conifers is the inability of induced shoots to form roots, e.g. *Pinus taeda* (Mehra-Palta et al. 1978), *Picea abies* (Von Arnold 1979), *Pinus pinaster* (David 1979), *Abies balsamea* (Bonga 1981), *Pinus*

sylvestris (Thompson and Zaerr 1982), *Larix decidua* (Bonga 1984), *Pinus lambertiana* and *Pseudotsuga menziesii* (Gupta and Durzan 1985). In this study, however, the use of the best treatment described above for rooting of the induced shoots from the juvenile material caused only limited rooting (16%) in the shoots produced on mature tissues. A low rate of rooting (11%) has also been reported with induced shoots from mature tissues of *Thuja plicata* (Coleman and Thorpe 1977). This may indicate that some factor other than growth substances limits root induction, and this merits attention in the future.

Development of an adventive root system and mycorrhizae formation

It is considered normal to see a delay (in this case 2 months) before the establishment of mycorrhizae (Faye et al. 1980). This delay may depend on several factors such as the presence of a suitable inoculum in the soil and the secretion of carbohydrates by the roots. The latter may well depend on the overall carbon balance of the plantlets (Bjorkman 1970, Hacskeylo 1973, Hayman 1974), so that factors like the root:shoot interaction are likely to play a part. It is evident from the data obtained here that mycorrhizal formation was considerably reduced on those roots systems that had been induced at high IBA (2 mg l^{-1}) or high BAP (0.5 mg l^{-1}). In fact, this was the case with mycorrhizal formation on rooted cuttings of *Pinus halepensis* (Gay 1982), and *Pinus pinaster* (Rancillac et al. 1982).

Survival and performance of plantlets in vivo

Data from this study demonstrate a high rate of shoot growth on induced root systems which originated on relatively low levels of auxin or cytokinin. Growth is considerably inhibited in roots initiated on high levels of auxin or cytokinin. It is of interest to note a close

correlation between shoot growth performance and the presence of mycorrhizae, and this may be due to the beneficial effects of ectomycorrhizae symbiosis on the host plant (Hacskeylo 1973, Harley and Smith 1983, Dixon and Marx 1984) especially in the nutrient-poor soils used in this study.

In fact, events such as poor shoot growth, and subsequent mortality of the plantlets reflect obvious physiological problems that might arise from a poor vascular connection between the induced shoot and root systems (Biondi and Thorpe 1982_a, Rancillac et al. 1982). Anatomical evidence supporting this conclusion has been reported with rooting *in vitro* of *Pinus sylvestris* (Gronroos and Von Arnold 1985). It is worthwhile observing that such events usually occurred on plantlets initially rooted under a regime of high auxin and cytokinin as in this study. Therefore, there may be important direct influences of the exogenous growth substances initially employed for root induction on the subsequent development and performance of the regenerated plantlets.

It seems evident that the developmental process is rather a biphasic phenomenon; the first stage is the initiation of organ primordia, followed by a second stage of stem bud elongation or root development. It is apparent that auxins and cytokinins (and presumably other constituents too) play a key role in regulating the first stage. However, the removal of these growth substances is critical to ensure further growth and development in the second phase. Both the responses of budding and rooting varied according to the type and concentration of cytokinin and auxin applied. The apparent efficiency of either BAP for bud production and NAA for root formation is likely to be a

function of their availability at a certain concentration in an active form (Jacobsen 1983). Also it is possible that BAP can act by inducing the production of a natural cytokinin within the tissues, thus working indirectly to stimulate the development process (Zaerr and Mapes 1982). The establishment of an effective and uniform root system on shoots grown *in vitro* is critical to the production of mass clonal material for commercial purposes (Mott 1981, Sommer and Caldas 1981). The results of this study underlined the need for care in choice and application of the growth factors for the initial growth induction to ensure not only good induction of buds or roots but also effective shoot elongation and successful performance of the regenerated plantlets after transplantation to soil.

4.4 INFLUENCE OF EXPLANT SOURCE

(i) Regeneration of plantlets

It is clear that the different explants of *Pinus brutia* used are capable, under appropriate culture conditions, of embarking on a course of organ induction and subsequent plantlet formation. *De novo* initiation of buds is relatively easy from cells manifesting a great morphogenic plasticity (embryonic, cotyledonary, and seedling materials). Importantly, it is possible to maintain bud and shoot production with time. Shoot production of up to 152 per clone with an average of 55 shoots per embryo was obtained over 24 weeks, and even after that time the induced meristematic tissue mass was still healthy and presumably capable of producing many more shoots, however, the limits of production have not been investigated (Abdullah et al. 1985). Also, following the optimum sequence of treatments in the seedling-needle culture system it was possible to produce 44 shoots per seedling within 18 weeks (32 plantlets per seedling within 26 weeks). Similar success has been reported with a few conifers (e.g. *Pinus radiata* (Aitken et al. 1981, Horgan and Aitken 1981, Smith 1986), *Pinus taeda* (Mott and Amerson 1981_a), *Picea mariana* & *Picea glauca* (Rumary and Thorpe 1984). Perhaps failure in maintaining the capability for continuous bud production may account for the poor yield of plantlets from other conifers *in vitro* (e.g. Ball 1950, Sommer et al. 1975, Cheng 1976, Coleman and Thorpe 1977, Webb and Street 1977, Cheng 1979, Bornman and Jansson 1980, Konar and Singh 1980, Minocha 1980, Mott and Amerson 1981_b, Von Arnold 1982_b, Bornman 1983, Bonga 1984, Patel and Thorpe 1984, Patel et al. 1986). Adventitious shoot initiation is

seasonal to some extent, in that the method requires the explants to be in a specific physiological and morphological state. This is evident in results obtained here and elsewhere (Reilly and Brown 1976, David et al. 1982, Bornman 1983, Durzan 1984_b). In fact, the negative effect imposed by the physiological age of the donor trees can be more pronounced if the differentiation has progressed too far (Jansson and Bornman 1980, 1980, Bornman 1983, Aitken and Horgan 1981, Bonga 1982_b). Perhaps this is the main bottleneck in exploitation of the totipotency of cells from older trees. Contradictory results emerging from other recent studies (Mott et al. 1977, Patel and Berlyn 1982, Von Arnold 1982_b, Papes et al. 1983, Hakman et al. 1984, Patel and Thorpe 1984, Renfroe and Berlyn 1984) may cast doubts on the genetic stability in these cultures.

The activation of preformed quiescent meristems in either seedling or mature plant material has proved useful for the micropropagation of Calabrian pine. However, the developmental potential of these dormant meristems is rather suppressed by other physiological factors. The production of the shoots in this system was maximized by the simultaneous removal of the apical bud (abolition of apical dominance, Kramer and Koslowski 1979) and the addition of cytokinin (Wickson and Thimann 1958). The efficiency of this procedure is also effective with explants taken from older (6 month) plants of *Pinus brutia*. This was previously shown to be the case for axillary bud production on seedlings of *Pseudotsuga menziesii* (Boulay 1976; 1979_a). However, in *Pinus pinaster*, a slow and reduced development has been recorded in buds produced on explants taken after the cotyledon stage (David et al. 1978) and a poor survival rate was reported with isolated shoots

developed from axillary buds on hypocotyl explants of *Pinus nigra* (Jelaska et al. 1982). In this study, production was not continued beyond the stage described here, but, with both techniques of shoot multiplication, it seems likely that successive generations could be achieved in which the shoot number would increase geometrically. Thus, by applying this procedure it should be possible to produce many thousands of shoots within one year. It seems that the time taken for axillary shoot production in the system described here is much shorter than with other systems. This is clearly advantageous for achieving the goal of commercial production (Sharp and Evans 1981, Thorpe and Biondi 1984).

Certainly the utilization of mature explants is most important when it is required to select trees of superior quality for propagation. Mature trees have been micropropagated via the actively growing parts (Bonga 1977). Also results from this study and others (Mehra-Palta et al. 1978, David 1979, Torbio and Pardos 1982, Gupta and Durzan 1985) indicate that the dormant meristems can be useful material. There seems to be a common requirement for an exogenous supply of cytokinin in the culture of these explants, but the main difficulty encountered in the maintenance of a healthy culture is oxidative browning (Von Arnold 1979, Gupta et al. 1980, Tisserat 1984, Von Arnold and Eriksson 1986). Unfortunately, some of the soak treatments reported in chapter 3 which are effective in reducing browning are quite unsuitable because they do not support the development of the dormant meristems nor the induction of adventitious buds on seedling needles. Indeed, there have been conflicting reports concerning the efficacy of such procedures; Von Arnold and Eriksson (1979_b) found no differences in developmental

responses between soaked and unsoaked resting buds of *Picea abies*, whereas soaking treatments have been reported to be critical in promoting the growth of embryonic shoots of *Abies balsamea* (Bonga 1977; 1981). Perhaps the most significant finding reported here is that SDD effectively prevents browning of the cultured fascicles, and also appears to stimulate the effect of applied cytokinins on shoot-bud formation. SDD has been recognized for some time as a substance which inhibits the activity of the copper-containing enzyme, phenoloxidase which is involved in the browning of *Picea abies* callus (Reinert and White 1956, Durzan et al. 1973). It is thought that SDD acts by chelating the copper which is essential for the activity of phenoloxidase, or it may prevent further synthesis of the oxidase by competing for copper (Smith 1968, Lerch 1981).

The inclusion of insoluble PVP (5 mg l^{-1}) also reduced browning due to the accumulation of polyphenols, but reduced the amount of regeneration. Polyamide can react with phenols and does restore enzyme activity; it absorbs the phenols through hydrogen-bonding, thus preventing their oxidation (Goldstein and Swain 1965, Loomis and Battaile 1966). A sequence of SDD treatment followed by PVP produced the best results in this study, which may reflect the fact that the double action of chelating the copper and absorbing the phenols is most effective. In fact, this approach might also be used to avoid possible copper deficiency in the case of prolonged cultivation on SDD-medium. Activated charcoal, at the level tested in this study, apart from its stimulating effect on the elongation of the basal part of the cultured fascicles, was not effective in reducing browning.

The time of year at which explants are removed and placed in culture

can influence their regenerative response (Boulay 1979_a, Bonga 1981, Selby and Harvey 1985). This is in contrast to the induction of adventitious buds on some conifer needles which requires the needles to be in an immature, relatively undifferentiated state (Yeung et al. 1981, David et al. 1982, David 1982, Bonga 1982_b, Bornman 1983). In this study the high frequency of single shoots formed on the cultured fascicles of *Pinus brutia* is dependent upon the advanced state of development of these fascicles. This is presumably because a well-developed meristematic region within the fascicle is required from which the shoots can be produced. It is also notable that explants collected in July are less prone to browning (Christiansen and Fønnesbech 1975, Hutchinson 1982).

Plantlets have been obtained from mature trees of *Sequoia sempervirens* (Boulay 1979_b), however, this was accomplished using sprouts taken from 50 year-old trees, and the capacity for sprouting is rare in gymnosperms, particularly in pine species. Hence, the most suitable type of explant must be determined for each species or perhaps each genus, as the results obtained here and elsewhere suggest (Bonga 1977; 1981; 1984, Mehra-Palta et al. 1978, David 1979, Von Arnold and Eriksson 1979_b, Thompson and Zaerr 1982, Toribio and Pardos 1982, Gupta and Durzan 1985).

(ii) In vivo performance of the plantlets

After a year of transfer into the soil, the plantlets which had been adventitiously propagated, using the best rooting protocol outlined earlier in this discussion, displayed serious problems such as plagiotropism, and unbalanced growth between root and shoot. Similar observations have been reported with adventitious micropropagules of

Pinus taeda (McKeand and Allen 1984, McKeand and Frampton 1984) and *Pseudotsuga menziesii* (Timmis and Ritchie 1984). However, plagiotropic growth is a characteristic of rooted cuttings taken from older trees (Mott 1981, Bonga 1982_a). It seems therefore that these plantlets, though they are of juvenile material-origin, behave like the mature plants. Perhaps the early development of needle fascicles on the adventive plantlets of *Pinus brutia* in this study, in *Pinus taeda* (Leach 1979), and in *Pinus radiata* (Aitken and Gleed 1984) would strengthen this view. In fact, abnormalities and variations in culture have been suggested to be induced by components of culture medium, particularly the exogenous growth substances (Durzan 1984_b). Though, some of the variations can be related to the choice of the explant over the life cycle of the plant (Durzan 1984_a, Timmis and Ritchie 1984). In *Pinus brutia*, both advanced maturation and high variation were more pronounced in the adventitious rather than the axillary plantlets, which may be related to the starting plant material. Indeed, acceleration of the maturation process may be desirable for the breeders, also some of the variation induced may be useful in reducing fungal infections of plantlets (McKeand and Frampton 1984). A long juvenile phase might be preferred if plants are to be cloned. More importantly, it seems possible that adventitious plantlets, being so abnormal, are unable to express properly the genetic potential of the parent (Timmis and Ritchie 1984). This is a very important consideration in the tissue culture propagation of clones, and we assume from results of this trial that the mode of axillary plantlet formation is most likely to avoid at least the above problems, and so to attain maximum similarity to *in vivo* performance of seedlings.

According to McKeand and Weir (1984) the elimination of genetic variation among cloned plantlets is expected to increase uniformity and ease stand management problems.

Slow growth of plantlets *in vivo* has been reported to be mainly related to insufficient nutrient uptake due to their poor root systems compared to those of seedlings (McKeand and Allen 1984). Results of this study confirmed that the quality of both the root and the plantlet can be improved by establishing a proper rooting protocol for cultured species. Again however, physiological disorders in plantlets that arise from improper shoot/root interaction might also be another cause for the poor growth shown by adventive plantlets. Disappointing performance *in vivo* recently reported with clones of conifer trees via embryonic tissues (McKeand and Frampton 1984, Timmis and Ritchie 1984) seems to have resulted from the same cause. These results reveal that the plant material employed is critical in determining the subsequent performance of tissue cultured plantlets *in vivo*.

4.5 REGULATION OF THE DEVELOPMENT PROCESS

Anatomical and physiological considerations

The initiation of meristematic tissues on various types of explants, either of *Pinus brutia* or other conifers, is dependent on the degree of differentiation. Under the influence of exogenous cytokinins those tissues in which mitotic activity still exists (Yeung et al. 1981, David et al. 1982) become meristematic. The target sites or cells are of epidermal or sub-epidermal origin in the embryos and cotyledons (e.g. Mott et al. 1977, Cheah and Cheng 1978, Von Arnold and Eriksson 1978, Tranvan 1979, Aitken et al. 1981, Yeung et al. 1981, Abdullah et al. 1985), presumably, because these cells are still in an embryonic state of growth. Even when young, pine needles have well differentiated epidermal and hypodermal layers with thick cell walls (Mirov 1967, Mott et al. 1977) which may explain why bud induction has often been restricted, as evident in *Pinus brutia*, to the needle's base where the mesophyll cells still manifest mitotic activity (the needle elongation area) (Reilly and Brown 1976, Mott et al. 1977, Jansson and Bornman 1980, David et al. 1982, Bornman 1983). After the target cells have commenced division and dedifferentiation the formation of a specific type of tissue (with densely cytoplasmic cells and large nuclei) called a meristemoid or a meristematic zone can be observed (e.g. Thorpe 1980, David 1982). This is the prerequisite for *de novo* initiation of bud primordia leading to bud formation.

Histological studies on *Pinus brutia in vitro* reveal that 3 weeks on a cytokinin-containing medium is necessary for the formation of desired meristematic activity. Results with *Pinus radiata in vitro* have shown

a similar incubation time is required to ensure a maximum rate of shoot formation (Biondi and Thorpe 1982_a, Villalobos et al. 1984_a). The data obtained here in this investigation also show that, irrespective of the mode of cytokinin application, 8 weeks are required for the induction of buds. These results may account for the low production of buds obtained on the needles of *Pinus brutia* pulse-treated with cytokinin. Presumably, the developmental path would be shorter using an organised system as the starting material (Murashige 1974). The anatomical evidence presented here shows this is indeed the case with *Pinus brutia*. The primary axillary buds that develop after culture arise directly from existing immature buds without an intervening callus stage, and both the primary axillary shoots and secondary buds were formed within 6 weeks. In previous reports (e.g. Boulay 1976, Bonga 1977, David et al. 1978, Jelaska et al. 1982) the exact origin of the buds is in some doubt. However, in this study it is clear that the secondary shoots arise directly from the primary shoots and not from callus, although some callus production occurs at the cut end of the explant.

It seems likely that the magnitude of the induced response in conifers is generally controlled by the cytokinin:auxin balance, as described in tobacco by Skoog and Miller (1957). Indeed, substantial progress has been made in the last decade, through careful use of growth substances, in the application of aseptic techniques for the propagation of crop plant. Such progress has unfortunately not contributed to an understanding of the action of hormones in the developmental process. Indeed, an understanding of the underlying control of the developmental process is still a long way off (Yeoman

1986). This is in part related to the lack of information regarding the status of endogenous hormones, and of the mechanism by which growth substances induce a developmental switch (Thorpe 1980, Zaerr and Mapes 1982, Thorpe and Biondi 1984). In this connection the recent reports on the rate of macromolecular synthesis (Villalobos et al. 1984_b), uptake kinetics (Vogelmann et al. 1984, Von Arnold and Eriksson 1985), and the suggested mechanism of cytokinin action via the nucleotide (Staden et al. 1986) may be of significance. Even this does not guarantee an explanation of the developmental phenomenon. Perhaps too much emphasis has been given to the role of growth substances in the regulation of development processes (Trewavas 1982; 1983, Durzan 1984_b, Bornman 1985). This preoccupation has tended to divert attention from the fact that development is controlled by a multiplicity of interacting factors and conditions (Steward 1983). It is now evident that the switch in the initiation programme is affected by the photomorphogenic action of light and cytokinin (Villalobos et al. 1984_a), and of equal importance is the photoperiodic requirement (Abdullah et al. 1985, Patel and Thorpe 1984, Patel et al. 1986). Also evidence which has emerged from this study and others (David et al. 1978, Bornman 1983, Abdullah et al. 1985) favours the involvement of nutrients in the determination of organogenic responses. Yet it is insufficient to consider the effect of cytokinin apart from that of the nutrient medium, as there may be strong interactions (Zaerr and Mapes 1982, Mott and Amerson 1984). No evidence is as yet available in this respect. However, the region of the tissue in which the cells are activated is thought to be influenced by physiological gradients of metabolites (more likely hormones and nutrients) moving out of the

medium and possibly from the surrounding cells into the actively dividing cells (Thorpe 1980, Zaerr and Mapes 1982, Staden et al. 1986). Taking this view the regulation of sink and source activities by the nutrients and hormones would be critical for the development pattern. Perhaps the documented role of nutrients, light and temperature in this study and those by others (Reilly and Brown 1976, Von Arnold 1979_b, Debregh 1983, Von Arnold 1984, Rumary and Thorpe 1984, Villalobos et al. 1984_a, Patel and Thorpe 1984, Abdullah et al. 1985). may allow some deduction to be made with regard to the environmental control of organogenesis.

Again, it should be recognized from this study and from the available literature (Bornman 1985, Durzan 1984_a; 1984_b, Bonga 1982_a; 1982_b) that the developmental fate of any given cell is determined by its state of differentiation which is also dictated by the age of the donor plant. The gradual failure of target cells to react to morphogenic information carried by the plant hormone molecules (Jacobsen 1983, Zajaczkowski et al. 1983) may be related to a decline in sensitivity to growth substances (Trewavas 1982; 1983) and is presumably a normal feature of cell aging.

Nevertheless, the nature of the signal that leads to the activation of certain cells to begin their altered pathway towards dedifferentiation and organogenesis is still not clear. Nor, whether this may be a hormonal signal of some sort (Thorpe 1980, Zaerr and Mapes 1982) or rather a possible mechanism which could influence the response of cells to the growth substances (Durzan 1984_a, Firn 1986). The search for triggers and markers of morphogenesis proceeds slowly (Yeoman 1986), and perhaps we need to learn much more about the

metabolic and genetic switches that control development (Bonga 1982_a;
1982_b).

4.6 GENERAL CONCLUSION AND PROSPECTS

The use of vegetative propagation offers the means to capture and transfer to the next generation all the genetic potential of the donor tree. This includes both the additive and non-additive genetic effects (Bonga 1982_a; 1982_b, Libby 1986). The classic methods of vegetative propagation seemed to be of limited value due to the low efficiency and difficulties in propagating mature trees (Girouard 1974, Libby 1974; 1979; 1986, Yeoman et al. 1978, Copes 1980, Bonga 1982_a, Yeoman 1984). Tissue culture methods have been seen as an obvious choice for the development of tree improvement programmes, but their usefulness as an alternative, at least in the near future, is still uncertain. This is particularly due to the fact that despite 35 years of continuous effort the *in vitro* methods developed so far still suffer from both biological and economical problems. Success, so far, has been limited to juvenile tissues (such as embryos and seedlings) of unknown phenotypes. A reliable procedure for plantlet regeneration has rarely been attained except for few species (e.g. *Pinus radiata* (Aitken and Gleed 1984; Smith 1986), *Pinus taeda* (Mott and Amerson 1984; McKeand and Frampton 1984), *Pseudotsuga menziesii* (Timmis and Ritchie 1984), *Pinus pinaster* (David et al. 1982; Rancillac et al. 1982)). This is partially because the requirement for each stage of organogenesis must be empirically determined, so the establishment of an efficient procedure requires a great deal of effort which means more time and money invested. So far an economic process for *in vitro* propagation of juvenile explants has only been achieved with *Pinus radiata* (Thorpe 1985, Smith 1986). Furthermore, except for a few cases, success has been restricted to the induction of adventitious shoots from non-meristematic explants,

despite the argument that more variation is likely to occur in such populations (Hussey 1978, Vasil and Vasil 1980, Sharpe and Evans 1981). Recently, reports have tended towards the conclusion that adventitious cultures of gymnosperms are rather stable (Papes et al. 1983, Renfroe and Berlyn 1984, Hakman et al. 1984), but stability at the diploid level does not exclude chromosomal aberrations or mutations (Berlyn et al. 1986). Of course, cloning via tissue culture methods should not increase genetic variability, otherwise it would lose its major attraction. Nevertheless, the potential for improving forest trees by exploiting the non-additive genetic gain can be considerable, as variations due to this genetic effect can exceed 50% (Zobel and Talbert 1984, Hasnain and Cheliak 1986). In this regard, cloning via tissue culture holds great promise as has been reported with *Pinus taeda* (McKeand and Weir 1984), and *Pinus radiata* (Aitken and Gleed 1984). But these variations should be evaluated both for their virtues and limitations (Durzan 1985, Yeoman 1986).

Perhaps some of the problems we are facing now is a result of the over enthusiastic use of exogenous growth substances in manipulating developmental patterns. The outcome of the present study would suggest that consideration should be given to establish a sequential treatment in which both a high rate of production and good *in vivo* performance of the micropropagules are attained. Here the culture environment and the explant source are of paramount importance. Unfortunately, these aspects have not as yet been properly considered, which accounts for the low efficiency of *in vitro* procedures of many species. Indeed, before these procedures can be justified, better control over the organogenetic process for mass production is of high priority.

Certainly, if tissue culture techniques are to be valuable it is essential to produce plantlets from trees old enough to manifest their genetic worth. In many gymnosperms this has been difficult or impossible via non-meristematic explants. Results of intense studies using the meristematic parts of these trees represents real progress, but the successful rooting of the shoots produced is still an incompleated task. We are again facing the old problem encountered in vegetative propagation of mature trees by rooted cuttings. Therefore, until this key problem can be overcome the utility of the *in vitro* methods developed remain limited. Also it is important to demonstrate that the micropropagules are truly cloned, i.e. true to type.

Economically, the production costs of micropropagules of gymnosperms, even with the best procedure developed in New Zealand for *Pinus radiata*, is still ten times higher than with seedlings, but in Canada, using *Pseudotsuga menziesii*, the cost differential is expected to be less (Hasnain and Cheliak 1986). However, the added genetic gain should outweigh the increasing cost of tissue cultured trees. Also direct plantlet formation via embryogenesis has been aimed at to reduce the cost, but in gymnosperms, we are along way from achieving somatic embryogenesis (Durzan 1985, Thorpe 1985).

The outlook seems to be that application of tissue culture techniques in forestry is still in its infancy and efforts must be expanded to realize the full potential of this fast developing technology (Karnosky 1981, Durzan 1985, Thorpe 1985, Yeoman 1986). While somatic embryogenesis might provide the breakthrough to produce bulk material at reduced cost, protoplast fusion may offer a new avenue for achieving substantial genetic gain, but unfortunately neither of

these methods are as yet workable with forest trees. It is apparent, in the immediate future, that clonal propagation via shoot proliferation is the most only available technique.

Calabrian pine in vitro

The traditional methods used to improve the quality of *Pinus brutia* via the establishment of seedling orchards from seeds of 'plus trees' is a long term programme, but is also an attractive approach for maintaining a broader genetic base. An outstanding genotype will appear only occasionally in such an orchard, whereas it can be reproduced many times in a clonal orchard. Unfortunately, Calabrian pine is a difficult species to propagate asexually even from juvenile parents. In this study, however, whole plantlets can be regenerated *in vitro* using various explants which means that a new approach to the genetic improvement of *Pinus brutia* has become available. The outline of the scheme developed in this study, and how it can be fitted to a conventional tree improvement programme, is illustrated in Fig. 4.1. The use of juvenile explants of conifers has a potential application for the *in vitro* regeneration of plants from scarce and costly seeds derived from controlled pollination (Bornman 1983). Also the use of embryonic (A) and seedling (B) tissues as a starting point seems to be useful to build up the base line information required for developing and refining more reliable procedures for *in vitro* culture of Calabrian pine and other conifers. The method developed for mass production of juvenile-origin (A and B) plantlets can be a promising means of extending the cloning of the best individuals (3) selected in the seedling orchard. However, the problem imposed by the physiological ageing of these materials would be the limiting factor. Perhaps the

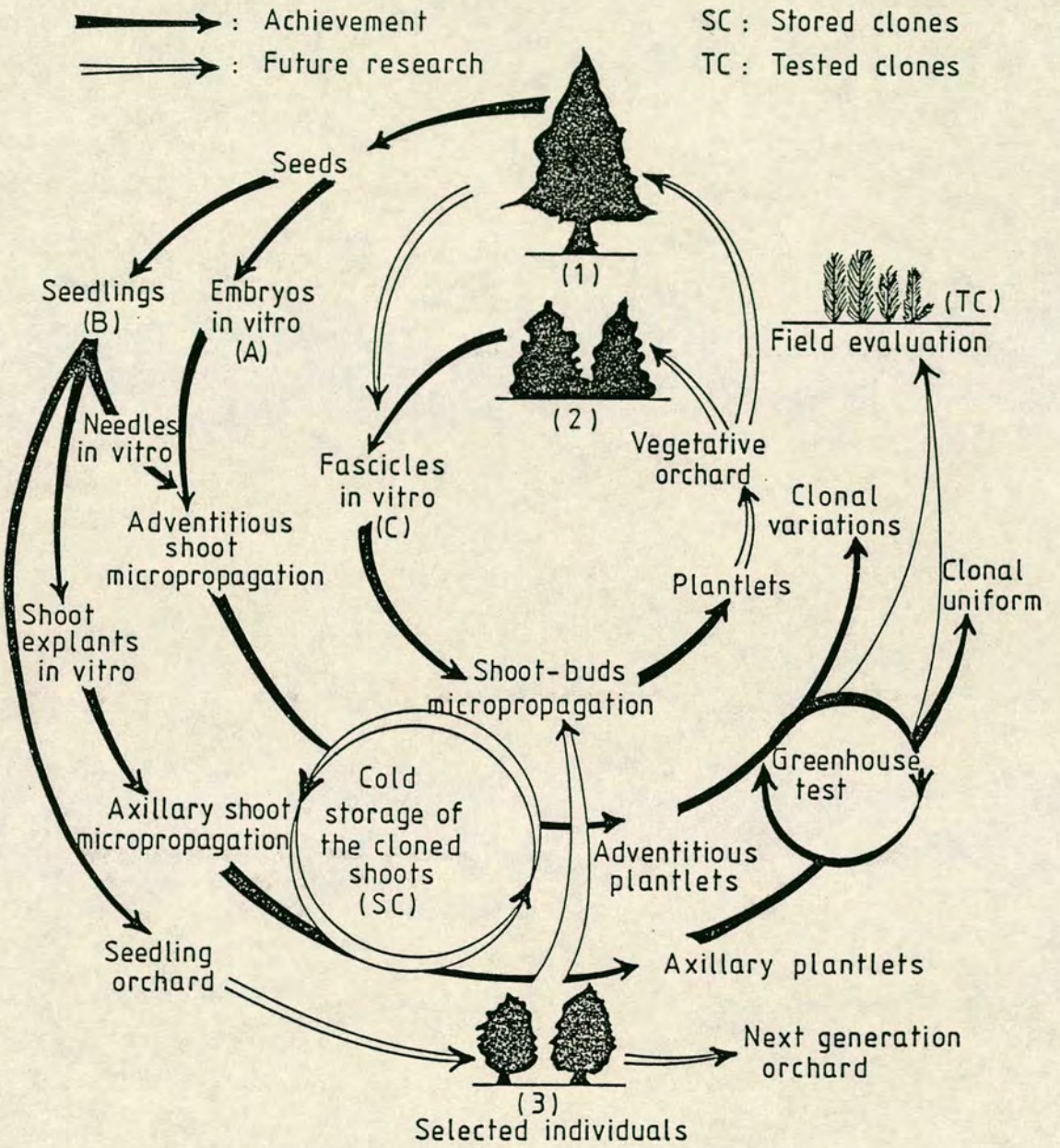


Fig. 4.1 The outlined programme (both the achievements and the prospects) and fitting of cloning *Pinus brutia* via tissue culture techniques with a conventional tree improvement.

combination of micropropagation of clones and long term cold storage of the produced clones (SC), whilst the same clones (TC) are being field tested, might offer a way around this obstacle. So far, with *Pinus brutia in vitro*, a high multiplication rate of shoots has been achieved in this study, but the cold storage aspect has not been investigated. The preliminary results obtained with cold stored shoots of *Pinus radiata* is encouraging (Smith 1986), although it is still uncertain if the shoots in cold storage will remain unchanged and juvenile (Aitken and Gleed 1984) nor if such process can be operated at a reasonable cost.

It is important to produce plantlets that are essentially uniform within a clone before a tissue culture technique will be of value for operational planting (Zobel and Talbert 1984). The present results would support the argument that *in vitro* propagation from established meristems is the best way to achieve this goal whilst providing an economical avenue to mass production (Murashige 1974, Hussey 1978, Mott 1981, Sharpe and Evans 1981, Yeoman 1986). On the other hand, somaclonal variations seem to be introduced via the adventitious pathway, which is also suggested for other species (Aitken and Gleed 1984, McKeand and Frampton 1984). But the value of these variations in *Pinus brutia*, if any, remain to be proven in the future.

The bulk of information gained in the juvenile *in vitro* pathway (A and B) is also critical in overcoming the practical barriers to propagating the fascicles (C) from older trees (2). In fact, vegetative propagation of explants from this source (2) would be extremely important for the improvement programme of *Pinus brutia*, as it would speed up the selection in this highly heterozygous species.

In the current strategy of maximum possible gain for forest improvement, both sexual reproduction and vegetative multiplication must be used (Zobel and Talbert 1984). The fascicle propagation pathway is of great potential value because it does permit the copying of unique individuals (3) within a generally superior family in the seedling orchard after a field test, thus giving a higher level of gain for the traits of interest (both growth rate and form in Calabrian pine) than the average value of the entire family. The next generation orchard would provide the diverse genetic base required for further genetic gain. This system offers possibilities for propagating even mature trees (1), whereby clonal vegetative orchards would be an attractive option, but it requires further work to attain the required level of reliability. The merit of the schemes outlined here in terms of the relative magnitude, and timing, of the gain would be greater than with the traditional method. However, a combination of both methods is essential to achieve the long term objective.

Bibliography

- Abdullah, A.A., Yeoman, M.M. & Grace, J. (1985).** *In vitro* adventitious shoot formation from embryonic and cotyledonary tissues of *Pinus brutia* Ten.. Plant Cell Tissue Organ cult. 5:35-44.
- Aitken, J., Horgan, K.J. & Thorpe, T.A. (1981).** Influence of explant selection on the shoot-forming capacity of juvenile tissue of *Pinus radiata*. Can. J. For. Res. 11:112-117.
- Aitken, J., Singh, A.P., Horgan, K.J. & Thorpe, T.A. (1982).** Cellular events influencing shoot formation in *Pinus radiata* cotyledons. In Proc. the 5th Intern. Cong. of Plant Tissue and Cell Culture, A. Fujiwara (ed.), pp. 175-176. The Japanese Assoc. Plant Tissue Cult., Maruzen Co. Ltd., Tokyo, Japan.
- Aitken, J. & Gleed, J. (1984).** Uses for micropropagation of juvenile *radiata* pine in New Zealand. In Proc. Intern. Symp. of Recent Advances in Forest Biotechnology, Michigan Institute of Biotechnology, pp. 47-57. Traverse City, Michigan.
- Amerson, H.V. & Mott, R.L. (1982).** Improved rooting of Western white pine shoots from tissue cultures. For. Sci. 28(4):822-825.
- Amos, R.R. & McCown, B.H. (1981).** Micropropagation of member of the coniferae. J. HortScience 16(3):453.
- Anders, O. & Kettaneh, M.S. (1969).** Forest Trees in Iraq. Mosul Univ. Press, Ninevah, Iraq.
- Anderson, L. & Wolter, K.E. (1966).** Cyclitols in plants: Biochemistry and physiology. Ann. Rev. Plant Physiol. 17:209-222.
- Ball, E.A. (1950).** Differentiation in a callus culture of *Sequoia sempervirens*. Growth 14:295-325.
- Berlyn, G.P., Deck, R.C. & Renfroe, M.H. (1986).** Tissue culture and the propagation and genetic improvement of conifers: Problems and possibilities. Tree Physiol. 1:227-240.
- Bjorkman, E. (1970).** Forest tree mycorrhiza- the conditions for its formation and significance of tree growth and afforestation. Plant and Soil. 32:589-610.
- Biondi, S., & Thorpe, T.A. (1982).** Clonal propagation of forest tree species. In Proc. Intern Symp. on Tissue Culture of Economically Important Plants. A.N. Rao (ed.), pp. 197-204. COSTED & ANBS, Singapore.

- Biondi, S. & Thorpe, T. (1982_b).** Growth regulator effect, metabolite changes, and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. *Bot. Gaz.* **143**:20-25.
- Bonga, J.M. & McInnis, A.H. (1975).** Stimulation of callus development from immature pollen of *Pinus resinosa* by centrifugation. *Plant Sci. Lett.* **4**:199-203.
- Bonga, J.M. (1977).** Organogenesis in *in vitro* cultures of embryonic shoots of *Abies balsamea* (balsam fir). *In Vitro* **13**:41-48.
- Bonga, J.M. (1981).** Organogenesis *in vitro* of tissues from mature conifers. *In Vitro* **17**:511-518.
- Bonga, J.M. (1982_a).** Vegetative propagation of mature trees by tissue culture. *In Proc. Symp. on Tissue Culture of Economically Important Plants.* A.N. Rao (ed.), pp. 191-196. COSTED & ANBS, Singapore.
- Bonga, J.M. (1982_b).** Vegetative propagation in relation to juvenility maturity, and rejuvenation. *In Tissue Culture in Forestry.* J.M. Bonga & D.J. Durzan (eds.), pp. 387-412. Martinus Nijhoff/Dr. W. Junk Publishers. The Hague, Netherlands.
- Bonga, J.M. (1984).** Adventitious shoot and root formation in tissue cultures of mature *Larix decidua*. *In Proc. Intern. Symp. of Recent Advances in Forest Biotechnology,* Michigan Institute of Biotechnology, pp. 64-68. Traverse City, Michigan.
- Boomsma, C.D. (1979).** Tree Planting Guide for South Australia, Bull. 12, D.J. Woolman. Government Printer, South Australia.
- Bornman, C.H. & Jansson, E. (1980).** Organogenesis in cultured *Pinus sylvestris* tissue. *Z. Pflanzenphysiol.* **96**:1-6.
- Bornman, C.H. & Jansson, E. (1982).** Regeneration of plants from the conifer leaf with special reference to *Picea abies* and *Pinus sylvestris*. *In Colloque Intern. Sur la Culture 'In Vitro' Des Essences Forestieres IUFRO Intern. Workshop Fontainebleau, France, Aug. 31st.- Sept. 4th. 1981.* pp. 41-53. AFOCEL, France.
- Bornman, C.H. (1983).** Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies in vitro* *Physiol. Plant.* **57**:5-16.
- Bornman, C.H. (1985).** Hormonal control of growth and differentiation in conifer tissues *in vitro*. *Biol. Plant.* **27**:249-256.

- Borthwick, A.W. (1899).** On interfoliar buds in pines. Trans. Proc. Bot. Soc. Edinburgh 21:154-158.
- Boulay, M. (1976).** Recherches sur la prolifération du Douglas par culture *in vitro*. AFOCEL Ann. Rech. Sylvic. 1976:84-145.
- Boulay, M. (1979_a).** Propagation *in vitro* du Douglas par micropropagation de germination aseptique et culture de bourgeons dormants. Etudes et recherches, AFOCEL, Domaine de l'Étançon, 77370 Nangis, France, 12:67-75.
- Boulay, M. (1979_b).** Multiplication et clonage rapide du *Sequoia sempervirens* par la culture *in vitro*. Micropropagation d'Arbres Forestieres, Ann. AFOCEL, 12:49-56, Nangis, France.
- Brown, C.L. & Sommer, H.E. (1975).** An Atlas of Gymnosperms Cultured *in vitro*: 1924-1974. Georgia Forest Research Council, Macon, Georgia.
- Brown, C.L. & Sommer, H.E. (1977).** Bud and root differentiation in Conifer cultures. Tappi 60(6):72-73.
- Chalupa, V. (1977_a).** Development of isolated Norway spruce and Douglas fir buds *in vitro*. Commun. Inst. For. Cech. 10:71-78.
- Chalupa, V. (1977_b).** Organogenesis in Norway spruce and Douglas fir tissue cultures. Commun. Inst. For. Cech. 10:79-87.
- Cheah, K. & Cheng, T.Y. (1978).** Histological analysis of adventitious bud formation in cultured Douglas fir cotyledon. Am. J. Bot. 65: 845-849.
- Cheng, T.Y. (1975).** Adventitious bud formation in culture of Douglas fir (*Pseudotsuga menziesii* Mirb. Franco.). Plant Sci. Lett. 5: 97-102.
- Cheng, T.Y. (1976).** Vegetative propagation of Western hemlock (*Tsuga heterophylla*) through tissue culture. Plant Cell Physiol. 17: 1347-1350.
- Cheng, T.Y. & Voqui, T. (1977).** Regeneration of Douglas-fir plantlets through tissue culture. Science. 198:306-307.
- Cheng, T.Y. (1977).** Factors affecting adventitious bud formation of cotyledon culture of Douglas fir. Plant Sci. Lett. 9:179-187.
- Cheng, T.Y. (1979).** Recent advances in development of *in vitro* techniques for Douglas fir. In Plant Cell and Tissue Culture Principles and Applications. W.R. Sharp, P. Larsen, E.F. Paddock

- & V. Raghavan (eds.), pp. 492-508. Ohio State University Press. Columbus.
- Christiansen, J. & Fonnesbech, M. (1975).** Prevention by polyvinylpyrrolidone of growth inhibition of Hamamelis shoot tips grown *in vitro* and of browning of the agar medium. *Acta Hortic.* 54:101-104
- Coleman, W.K. & Thorpe, T.A. (1977).** *In vitro* culture of Western redcedar (*Thuja plicata* Donn.). I. Plantlet formation. *Bot. Gaz.* 138(3):298-304.
- Copes, D.L. (1980).** Development of internal graft incompatibility symptoms in *Pinus radiata* Don.. *N.Z.J. For. Sci.* 10:367-380.
- Dallimore, W. & Jackson, A.B. (1954).** A Handbook of Coniferae, Edward Arnold (Publ.) Ltd., London.
- David, H., Isemukali, K. & David, A. (1978)** Obtention de plants de pin maritime (*Pinus pinaster* Sol.) à partir de brachyblastes ou d'apex caulinaires de très jeunes sujets cultivés *in vitro*. *C.R. Acad. Sci.* 287:245-248.
- David, A (1979)** Manifestations de diverses potentialités organogènes et micropropagation végétative chez le pin maritime (*Pinus pinaster* Sol.). *Ann. Rech. Sylvicoles, AFOCEL*, 12:57-75.
- David, A. (1982).** *In vitro* propagation of gymnosperms, *In Tissue Culture in Forestry.* J.M. Bonga & D.J. Durzan (eds.), pp.72-108. Martinus Nijhoff/Dr. W. Junk Publishers. The Hague, Netherlands.
- David, A., David, H. & Mateille, T. (1982).** *In vitro* adventitious budding on *Pinus pinaster* cotyledons and needles. *Physiol. Plant* 56:102-107.
- David, H., Jarlet, E. & David, A. (1984).** Effects of nitrogen source, calcium concentration and osmotic stress on protoplasts and protoplast-derived cell cultures of *Pinus pinaster* cotyledons. *Physiol. Plant.* 61:477-482.
- Davis, P.H. (1965).** Flora of Turkey, vol.1. Edinburgh Univ. Press, Edinburgh. Scotland.
- Debergh, P.C. (1983).** Effects of agar brand and concentration on the tissue culture medium. *Physiol. Plant.* 59:270-276.
- Devlin, R. (1975).** Functions of the essential mineral elements and symptoms of mineral deficiency. *In Plant Physiology*, pp.353-408. D. Van Nostrand Co., New York-London.

- Dixon, R.K. & Marx, D.H. (1984).** The role of mycorrhizae in forest biotechnology. In Proc. Intern. Symp. on Recent Advances in Forest Biotechnology, Michigan Institute of Biotechnology, pp. 126-138. Traverse City, Michigan.
- Douglas, T.J., Villalobos, V.M., Thompson, M.R., & Thorpe, T.A. (1982).** Lipid and pigment changes during shoot initiation in cultured explants of *Pinus radiata*. *Physiol. Plant.* 55:470-477.
- Duncan, D.B. (1955).** Multiple range and multiple F-tests. *Biometrics* 11:1-42.
- Durzan, D.J., Chafe, S.C. & Lopushonski, S.M. (1973).** Effects of environmental changes on sugar, tannins, and organized growth in cell suspension cultures of White spruce. *Planta* 113:241-249.
- Durzan, D.J. (1984).** Potential for genetic manipulation of forest trees: totipotency, somaclonal aberration and trueness of type. In Proc. Intern. Symp on Recent Advances in Forest Biotechnology, Michigan Institute of Biotechnology, pp. 104-125. Traverse City, Michigan.
- Durzan, D.J. (1984).** Special problems: Adult vs. juvenile explants. In Handbook of Plant Cell Culture, Crop Species. W.R. Sharp, D.A. Evans, P.V. Ammirato & Y. Yamada (eds.), pp. 471-503. Macmillan Publ. Co., New York-London.
- Durzan, D.J. (1985).** Biotechnology and the cell cultures of woody perennials. *The For. Chron.* 61(5):439-447.
- Faye, M., Rancillac, M. & David, A. (1980).** Determinism of the mycorrhizogenic root formation in *Pinus pinaster* Sol.. *The New Phytol.* 87:557-565.
- Fink, S. (1984).** Some cases of delayed or induced development of axillary buds from persisting detached meristems in conifers. *Amer. J. Bot.* 71(1):44-51.
- Firn, R.D. (1986).** Growth substance sensitivity: The need for clearer ideas, precise terms and purposeful experiments. *Physiol Plant.* 67: 267-272.
- Fosket, O.E. (1980).** Hormonal control of morphogenesis in cultured tissues. In Proc. 10th Intern. Conf. on Plant Growth Substances. F. Skoog (ed.), pp. 362-369. Springer-Verlag, Berlin-New York.
- Gamborg, O.L. & Shyluk, J.P. (1970).** The culture of plant cells with

- ammonium salts as the sole nitrogen source. *Plant Physiol.* **45**: 598-600.
- Gay, P.G. (1982).** Influence d'un champignon ectomycorhizien sur l'enracinement d'hypocotyles excisés de *Pinus halepensis* Mill. In Colloque Intern. sur la culture 'in vitro' des Essences Forestières, IUFRO Intern. Workshop, Fontainebleau, France, Aug. 31st.- Sept. 4th. 1981, pp. 269-276. AFOCEL, France.
- Girouard, R.M. (1974).** Propagation of spruce by stem cuttings. *N.Z. For. Sci.* **4**:140-149.
- Goldstein, J.L. & Swain, T. (1965).** The inhibition of enzymes by tannins. *Phytochem.* **4**:185-192.
- Gresshoff, P.M. & Doy, C.H. (1972).** Development and differentiation of haploid *Lycopersicon esculentum* (tomato). *Planta* **107**:161-170.
- Gronroos, R. & Von Arnold, S. (1985).** Initiation and development of wound tissue and roots on hypocotyl cuttings of *Pinus sylvestris* in vitro. *Physiol. Plant.* **64**:393-401.
- Gupta, P., Nadgir, A., Mascarenhas, A. & Jagannathan, V. (1980).** Tissue culture of forest trees: Clonal multiplication of *Tectona grandis* L. (teak) by tissue culture. *Plant Sci. Lett.* **17**:259-268.
- Gupta, P.K. & Durzan, D.J. (1985).** Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and Sugar pine (*Pinus lambertiana*). *Plant Cell Reports* **4**:177-179.
- Haberlandt, G. (1902).** Kultinversuche mit isolierten Pflanzellen. *Sber. Akad. Wiss. Wien* **111**:69-92.
- Hacskeylo, E. (1973).** Carbohydrate physiology of ectomycorrhizae. In *Ectomycorrhizae, Their Ecology and Physiology*. G.C. Marks & T.T. Kozlowski (eds.), pp. 207-230. Academic Press, New York-London.
- Haissig, B. (1974).** Influences of auxins and auxin synergists on adventitious root primordium initiation and development. *N.Z. J. For. Sci.* **4**:311-323.
- Hakman, I., Von Arnold, S. & Bengtsson, A. (1984).** Cytofluorometric measurements of nuclear DNA in adventitious buds and shoots of *Picea abies* regenerated in vitro. *Physiol. Plant.* **60**:321-325.
- Harley, J.L. & Smith, S.E. (1983).** Mycorrhizal Symbiosis. Academic Press London-New York.

- Hasnain, S. & Cheliak, W. (1986)** Tissue culture in forestry: Economic and genetic potential. *The For. Chron.* **62(4)**:219-225.
- Horgan, K. & Aitken, J. (1981)**. Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. *Physiol. Plant.* **53**:170-175.
- Hughes, K.W. (1981)**. *In vitro* ecology: exogenous factors affecting growth and morphogenesis in plant culture systems. *Environ. Exp. Bot.* **21**:281-288.
- Hunt, R. (1982)**. *Plant Growth Curves: The Functional Approach to Plant-Growth Analysis*. Edward Arnold, London.
- Hussey, G (1978)** The application of tissue culture to the vegetative propagation of plants. *Sci. Prog. (Oxford)* **65**:185-208.
- Hutchinson, J.F. (1982)**. *In vitro* propagation of apple using organ culture. *In Proc. the 5th Intern. Cong. on Plant Tissue and Cell Culture*, A. Fujiwara (ed.), pp.729-730. The Japanese Assoc Plant Tissue Cult.. Maruzen Co. Ltd., Tokyo, Japan.
- Jacquot, C. (1966)**. Plant tissues and excised organ cultures and their significance in forest research. *J. Inst. Wood Sci.* **16**:22-34
- Jacobsen, H.J. (1983)**. Biochemical mechanisms of plant hormone activity. *In Handbook of Plant Cell Culture, Techniques for Propagation and Breeding*, D.A. Evans, W.R. Sharp, P.V. Ammirato & Y. Yamada (eds.), pp. 672-695. Macmillan Publ. Co., New York-London.
- Jansson, E. & Bornman, C. (1980)** *In vitro* phyllomorphic regeneration of shoot buds and shoots in *Picea abies*. *Physiol. Plant.* **49**: 105-111.
- Jansson, E. & Bornman, C.H (1981)** *In vitro* initiation of adventitious structures in relation to the abscission zone in needle explants of *Picea abies*: anatomical considerations. *Physiol. Plant.* **53**: 191-197.
- Jansson, E. & Bornman, C.H (1983)**. Morphogenesis in dormant embryonic shoots of *Picea abies*: Influence of the crown and cold treatment *Physiol. Plant.* **59**:1-8.
- Jelaska, S., Kolevska-Pletikapic, B. & Vidakovic, M. (1982)**. Bud regeneration in *Pinus nigra* embryo and seedling tissue culture. *In Colloque Intern. Sur La Culture 'In Vitro' Des Essence Forst-*

- ieres, IUFRO Intern. Workshop, Fontainbleau, France Aug. 31st-Sept. 4, 1981. pp. 159-165. AFOCEL, Nangis, France.
- Karnosky, D.F. (1981).** Potential for forest tree improvement via tissue culture. *BioSci.* 31(2):114-120.
- Karnovsky, M. (1965).** A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* 27: 137A-138A.
- Kayacik, H. & Yaltirik, F. (1970)** General aspect of Turkish forestry. In *Plant Life of South-West Asia*, P.H. Davis, P.C. Harper & I.C. Hedge (eds.), pp. 284-291. Aberdeen Univ. Press, Scotland.
- Kevers, C., Coumans, M., Coumans-Gilles, M.-F. & Gaspar, Th. (1984).** Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. *Physiol. Plant.* 61:69-74.
- Konar, R.N. & Singh, M.N (1980).** Induction of shoot buds from tissue cultures of *Pinus willichiana*. *Z.Pflanzenphysiol.* 99:173-177.
- Kramer, P.J. & Kozlowski, T.T. (1979).** Internal factors affecting growth. In *Physiology of Woody Plants*, P.J. Kramer & T.T. Kozlowski (eds.), pp. 546-627. Academic Press, New York-London.
- Le Houérou, H. (1981)** Impact of man and his animals on Mediterranean vegetation. In *Ecosystems of The World: Mediterranean-type shrublands*, Vol. 11, F. Di Castri, D.W. Goodall, & R.L. Specht (eds.), pp. 479-521. Elsevier scientific Publ. Co., Amsterdam-Oxford-New York.
- Leach, G.N. (1979).** Growth in soil of plantlets produced by tissue culture, Loblolly pine. *Tappi* 62(4):59-61.
- Lerch, K. (1981).** Copper monooxygenases: tyrosinase and dopamine *B*-monooxygenase. In *Metal Ions in Biological Systems*, Vol. 13, Copper Proteins, H. Sigel (ed.), pp.143-186. Marcel Dekker, Inc. New York and Basel.
- Libby, W.J. (1974).** The use of vegetative propagules in forest genetics and tree improvement. *N.Z.J. For. Sci.* 4:440-447.
- Libby, W.J. (1979).** Clonal selection of forest trees. *Calif. Agric.* 33:8-9.
- Libby, W.J. & Rauter, R.M. (1984)** Advantages of clonal forestry. *The For. Chron.* 60:145-149.
- Libby, W.J. (1986).** Clonal propagation, the clones are coming. *J.*

- For. 84:37-42.
- Linsmaier, E. & Skoog, F. (1965).** Organic growth factor requirements of tobacco tissue culture. *Physiol. Plant.* 18:100-127.
- Loomis, W.D. & Battaile, J. (1966).** Plant phenolic compounds and the isolation of plant enzymes. *Phytochem.* 5:423-438.
- McKeand, S.E. & Allen, H.L. (1984).** Nutritional and root development factors affecting growth of tissue culture plantlets of Loblolly pine. *Physiol. Plant.* 61:523-528.
- McKeand, S.E. & Frampton, L.J. (1984).** Performance of tissue culture plantlets of Loblolly pine *in vivo*. In *Proc. Intern. Symp. of Recent Advances in Forest Biotechnology*, Michigan Institute of Biotechnology, pp. 82-91. Traverse City, Michigan.
- McKeand, S.E. & Weir, R. (1984)** Tissue culture & Forest productivity. *J. For.* 82:212-218.
- Mehra-Palta, A., Smeltzer, R.H. & Mott, R.L. (1978).** Hormonal control of induced organogenesis. Experiments with excised plant parts of Loblolly pine. *Tappi* 61:37-40.
- Michaelides, E.D. 1978.** Development of forestry in Cyprus. Nicosia Press, Cyprus.
- Minocha, S.C. (1980).** Callus and adventitious shoot formation in excised embryos of White pine (*Pinus strobus*). *Can. J. Bot.* 58: 366-370.
- Mirov, N.T. (1955).** Relationships between *Pinus halepensis* and other insignes pines of the Mediterranean region. *Bull. Res. Council. of Israel* 5D:65-72.
- Mirov, N.T. (1967).** The Genus *Pinus*. The Ronald Press Co., New York.
- Mirov, N.T. & Hasbrouck, J. (1976).** The Story of Pines. Indiana Univ. Press, Bloomington/London.
- Misson, J.P. (1982)** Induction of adventitious buds on buds of *Picea punens* grown *in vitro*. *Z.Pflanzenphysiol* 107:161-176.
- Mott, R.L., Smeltzer, R.H. & Mehra-Palta, A. (1977).** An anatomical and cytological perspective on pine organogenesis *in vitro*. In *Tappi Conf. Papers, Forest Biology/Wood Chemistry Conference*, pp.9-14. Madison, USA.
- Mott, R.L. (1981).** Trees. In *Cloning Agricultural plants via in vitro Techniques*, B.V. Conger (ed.), pp. 217-254. CRC Press

- Inc., Boca Raton/ Florida.
- Mott, R.L. & Amerson, H.V. (1981_a)**. A tissue culture process for the clonal production of loblolly pine plantlets. North Carolina Agric. Res. Ser. Tech. Bull. 271:1-14.
- Mott, R.L. & Amerson, H.V. (1981_b)**. Tissue culture plantlets produced from *Pinus monticola* embryonic materials. For.Sci. 27(2):299-304
- Mott, R.L. & Amerson, H.V. (1984)**. Role of tissue culture in Loblolly pine improvement. In Proc. Intern. Symp. of Recent Advances in Forest Biotechnology, Michigan Institute of Biotechnology, pp. 24-36. Traverse City, Michigan.
- Moulopoulos, C. & Bassiotis, C. (1963)** Investigations on crossability of some Mediterranean pines and the American red pine, In Proc. the World Consultation on Forest Genetics and Tree Improvement, vol.1, Section (2b/10), pp. 1-7. FAO, Stockholm.
- Murashige, T. & Skoog, F. (1962)**. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
- Murashige, T. (1974)**. Plant propagation through tissue culture. Ann. Rev. Plant Physiol. 25:135-166.
- Murashige, T. (1978)**. The impact of plant tissue culture on agriculture. In Frontiers of Plant Tissue Culture, T.A. Thorpe (ed), pp.15-26. Calgary Univ. Printing Services, Canada.
- Nahal, I. (1981)**. The mediterranean climate from a biological viewpoint. In Ecosystems of The World: Mediterranean-type shrublands Vol. 11, F. Di Castri, D.W. Goodall & R.L. Specht (eds.), pp. 63-86. Elsevier scientific Publ. Co., Amsterdam-Oxford-New York.
- Obata-Sasamoto, H., Villalobos, V. & Thorpe, T (1984)** ¹⁴C-Metabolism in cultured cotyledon explants of radiata pine. Physiol. Plant. 61:490-496.
- Papes, D., Jelaska, S. & Kolevska-Pletikapic, B. (1983)**. Chromosome behaviour in cultured cell populations of higher plants. In Kew Chromosome Conf. II, Kew 1-4 Sept. 1982, P.E. Brandham and M.D. Bennett (eds.), pp. 155-163. George Allen & Unwin Publ. London.
- Patel, K.R. & Berlyn, G.P. (1982)**. Genetic instability of multiple buds of *Pinus coulteri* regenerated from tissue culture. Can. J. For. Res. 12:93-101.

- Patel, K.R. & Thorpe, T.A. (1984). *In vitro* differentiation of plantlets from embryonic explants of Lodgepole pine (*Pinus contorta* Dougl. ex Loud.). *Plant Cell Organ Cult.* 3:131-142.
- Patel, K.R., Kim, H. & Thorpe, T.A. (1986). Plantlet formation in Pitch pine (*Pinus rigida* Mill.) by tissue culture methods. *For. Ecol. Manage.* 15:147-160.
- Quézel, P. (1981). The study of plant groupings in the countries surrounding the Mediterranean: Some methodological aspects. *In Ecosystems of the World: Mediterranean-type shrublands*, vol. 11. F. Di Castri, D.W. Goodall & R.L. Specht (eds.), pp. 87-93. Elsevier Scientific Publ. Co., Amsterdam- Oxford- New York.
- Rancillac, M., Faye, M. & David, A. (1982). *In vitro* rooting of cloned shoots in *Pinus pinaster*. *Physiol. Plant.* 56:97-101.
- Reilly, K. & Brown, C.L. (1976). *In vitro* studies of bud and shoot formation in *Pinus radiata* and *Pseudotsuga menziesii*. Georgia Forest Res. paper 86:1-9.
- Reilly, K. & Washer, J. (1977). Vegetative propagation of radiata pine by tissue culture: plantlet formation from embryonic tissue. *N.Z. J. For. Sci.* 7(2):199-206.
- Reinert, J. & White, P.R. (1956). The cultivation *in vitro* of tumour tissues and normal tissues of *Picea glauca*. *Physiol. Plant.* 9: 177-189.
- Renfro, M.H. & Berlyn, G.P. (1984). Stability of nuclear DNA content during adventitious shoot formation in *Pinus taeda* L. tissue culture. *Amer. J. Bot.* 71(2):268-272.
- Roland, J. (1978). General preparation and staining of thin sections. *In Electron Microscopy and Cytochemistry of Plant Cells*, J.L. Hall (ed.), pp. 1-60. Elsevier Publ. Co., Amsterdam.
- Rumary, C. & Thorpe, T.A. (1984) Plantlet formation in black and white spruce. I. *In vitro* techniques. *Can. J. For. Res.* 14:10-16.
- Schenk, R.U. & Hildebrandt, A.C. (1972). Media and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell culture. *Can. J. Bot.* 50:199-204.
- Scott, T.K. (1972). Auxins and roots. *Ann. Rev. plant Physiol.* 23: 235-258.
- Selby, C. & Harvey, 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. *The New Phytol.* 100:549-562.
- Sharp, W.R. & Evans, D.A. (1981).** Patterns of plant regeneration: opportunities for cloning or production of genetic variability. *Intern. Assoc. Plant Tissue Cult. NewsLett.* 35:2-8.
- Skoog, F. & Miller, C.O. (1957).** Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. 11th Symp. *Soc. Exp. Biol.* 11:118-131.
- Skoog, F. (1971).** Aspects of growth factor interactions in morphogenesis of tobacco tissue cultures. *In Les Cultures de Tissue de Plants. Colloq. Intern. CNRS* 193:115-136.
- Smith, D.L. (1968).** The growth of shoot apices and inflorescences of *Carex flacca* Schreb. in aseptic culture. *Ann. Bot.* 32:361-370.
- Smith, D.R. (1986).** Forest and nut trees: 1. Radiata pine (*Pinus radiata* D. Don). *In Biotechnology in Agriculture and Forestry 1, Trees I*, Y.P.S. Bajaj (ed.), pp.274-291. Springer-Verlag, Berlin-Hiedelberg-New York-Tokyo.
- Snedecor, G.W. & Cochran, W.G. (1980).** *Statistical Methods*, 7th edn., Iowa State University Press.
- Sommer, H.E., Brown, C.L. & Kormanik, P.P. (1975).** Differentiation of plantlets in longleaf pine (*Pinus palusteris* Mill.) tissue cultured *in vitro*. *Bot. Gaz.* 136:196-200.
- Sommer, H.E. & Caldas, L. (1981).** *In vitro* methods applied to forest trees. *In Plant Tissue Culture, Methods and Applications in Agriculture*, T.A. Thorpe (ed), pp.349-358. Academic Press, New York-London.
- Staden, J., Forsyth, C., Bergman, L. & Von Arnold, S (1986).** Metbolism of benzyladenine by excised embryos of *Picea abies*. *Physiol. Plant.* 66:427-434.
- Steward, F.C. (1983).** Reflections on aseptic culture. *In Handbook of Plant Cell Culture, Techniques for propagation and breeding*, D.A. Evans, W.R. Sharp, P.V. Ammirato, & Y. Yamada (eds.), pp. 1-10. Macmillan Publ. Co., New York-London.
- Stockman, L. (1980).** Availability and demand patterns for renewable resources-Forestry. *In Future Sources of Organic Raw Materials-*

- Chemrawn 1, L.E. Pierre & G.R. Brown (eds.), pp.41-49. Pergamon Press, Oxford.
- Stone, E.L. & Stone, M.H. (1943).** Dormant buds in certain species of *Pinus*. *Amer. J. Bot.* 30:346-351.
- Thomas, M., Duhoux, E. & Vazart, J. (1977).** *In vitro* organ initiation in tissue cultures of *Biota orientalis* and other species of Cupressaceae. *Plant Sci. Lett.* 8:395-400.
- Thompson, D.G. & Zaerr, J. (1982).** Induction of adventitious buds on cultured shoot tips of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco). In *Colloque Intern. Sur La Culture 'In Vitro' Des Essence Forestiers*, IUFRO Intern. Workshop, Fontainebleau-France, Aug. 31st- Sept 4, 1981, pp. 167-174. AFOCEL, Nangis, France.
- Thorpe, T.A. & Murashige, T. (1968).** Starch accumulation in shoot-forming tobacco callus cultures. *Science* 160:421-422.
- Thorpe, T.A. (1980)** Organogenesis *in vitro*: structural, physiological, and biochemical aspects, In *Intern. Rev. Cyto.*, Suppl. 11A, I. K. Vasil (ed.), pp.71-111. Academic Press, London.
- Thorpe, T.A. (1982).** Carbohydrate utilization and metabolism. In *Tissue Culture in Forestry*, J.M. Bonga & D.J. Durzan (eds.), pp. 325-368. Martinus Nijhoff/ Dr. W. Junk Publishers. The Hague.
- Thorpe, T.A. & Biondi, S. (1984).** Fiber and Wood: Conifers. In *Handbook of Plant Cell Culture, Crop Species*, W.R. Sharpe, D.A. Evans, P.V. Ammirato, & Y. Yamada (eds.), pp.435-467. Macmillan Publ. Co., New York- London.
- Thorpe, T.A. (1985).** Application of tissue culture technology to forest improvement. *The For. Chron.* 61(5):436-438.
- Timmis, R. & Ritchie, G.A. (1984).** Progress in Douglas-fir tissue culture. In *Proc. Intern. Symp. of Recent Advances in Forest Biotechnology*, Michigan Institute of Biotechnology, pp. 37-46. Traverse City, Michigan.
- Tisserat, B. (1984).** Date palm. In *Handbook of Plant Cell Culture, Crop Species*, W.R. Sharp, D.A. Evans, P.V. Ammirato & Y. Yamada (eds.), pp. 506-545. Macmillan Publ. Co., New York-London.
- Toribio, M. & Pardos, J.A. (1982)** *In vitro* organogenesis of *Pinus Sylvestris* L. tissues. In *Colloque Intern. Sur La Culture 'In Vitro' Des Essence Forestiers*, IUFRO Intern. Workshop,

- Fontainebleau-France, Aug. 31st- Sept. 4th. 1981. pp. 143-148 AFOCEL, Nangis, France.
- Tranvan, H. (1979).** *In vitro* adventitious bud formation on isolated seedlings of *Pinus sylvestris* L. Biol. Plant. 21(3):230-233.
- Trewavas, A. J. (1982).** Growth substance sensitivity: the limiting factor in plant development. Physiol. Plant. 55:60-72.
- Trewavas, A. J. & Cleland, R. E. (1983).** Is plant development regulated by changes in the concentration of growth substances or by changes in the sensitivity to growth substances? (Discussion Forum). Trends in Biochem. Sci. 8(10):354-357.
- Vasil, I. K. & Vasil, V. (1980).** Clonal propagation. In Intern. Rev. Cyto., Suppl. 11A., I. K. Vasil (ed.), pp. 145-173. Academic Press, New York-London.
- Villalobos, V. M., Leung, D. W. & Thorpe, T. A. (1984_a).** Light-cytokinin interaction in shoot formation in cultured cotyledon explants of radiata pine. Physiol. Plant. 61:497-504.
- Villalobos, V. M., Oliver, M. & Thorpe, T. A. (1984_b).** Cytokinin-induced switch in the development in excised cotyledons of radiata pine cultured *in vitro*. Physiol. Plant. 61:483-489.
- Vogelmann, T. C., Bornman, C. H. & Nissen, P. (1984).** Uptake of benzyladenine in explants of *Picea abies* and *Pinus sylvestris*. Physiol. Plant. 61:513-517.
- Von Arnold, S. & Eriksson, T. (1978).** Induction of adventitious buds on embryos of Norway spruce grown *in vitro*. Physiol. Plant. 44:283-287.
- Von Arnold, S. & Eriksson, T. (1979_a).** Induction of adventitious buds on buds of Norway spruce (*Picea abies*) grown *in vitro*. Physiol. Plant. 45:29-34.
- Von Arnold, S. & Eriksson, T. (1979_b).** Bud induction on isolated needles of Norway spruce (*Picea abies* L., Karst.) grown *in vitro*. Plant Sci. Lett. 15:363-372.
- Von Arnold, S. (1979).** Induction and development of adventitious bud primordia on embryos, buds and needles of Norway spruce (*Picea abies* (L.) Karst) grown *in vitro*. Abstracts of Uppsala. Dissertations from the Faculty of Science, Sweden: ACTA Univ. Uppsaliensis.

- Von Arnold, S. & Eriksson, T. (1981) *In vitro* studies of adventitious shoot formation in *Pinus contorta*. Can. J. Bot. 59:870-874.
- Von Arnold, S. (1982^a). Vegetative propagation of *Picea abies*. In Proc. the 5th Intern. Cong. of Tissue and Cell Culture, A. Fujiwara (ed.), pp. 753-754. The Japanese Assoc. Plant Tissue Cult., Maruzen Co. Ltd., Tokyo, Japan.
- Von Arnold, S. (1982^b). Factors influencing formation, development and rooting of adventitious shoots from embryos of *Picea abies* (L.) Karst. Plant Sci. Lett. 27:275-287.
- Von Arnold, S. & Eriksson, T. (1984). Effect of agar concentration on growth and anatomy of adventitious shoots of *Picea abies* (L.) Karst. Plant Cell Tissue Organ Cult. 3:257-264.
- Von Arnold, S. (1984). Importance of genotype on the potential for *in vitro* adventitious bud production of *Picea abies*. For. Sci. 30(2):314-318.
- Von Arnold, S. & Eriksson, T. (1985). Initial stages in the course of adventitious bud formation on embryos of *Picea abies*. Physiol. Plant 64:41-47.
- Von Arnold, S. & Eriksson, T. (1986). Forest and nut trees: 2. Norway spruce (*Picea abies* L.). In Biotechnology in Agriculture and Forestry 1, Trees I, Y.P.S. Bajaj (ed.), pp. 291-310. Springer-Verlag, Berlin-Heidelberg-New York-Tokyo.
- Weatherhead, M.A., Burdon, J. & 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. & Henshaw, G.G. (1979). Some effects of activated charcoal as an additive to plant tissue culture media. Z.Pflanzenphysiol 94:399-405.
- Webb, D.B. (1974) The Trial of Exotic Species in the Semi-Arid Zone of Iran. Oxford Univ. Press, Oxford.
- Webb, K.J. & Street, H.E. (1977). Morphogenesis *in vitro* of *Pinus* and *Picea*. Acta. Hortic. 78:259-269.
- Wickson, M. & Thimann, K.V (1958) The antagonism of auxin and kinetin in apical dominance. Physiol. Plant. 11:62-74.
- Winton, L. & Verhagen, S.A. (1977). Shoots from Douglas-fir cultures. Can. J. Bot. 55:1246-1250.

- Yamakawa, T., Kurahashi, O., Ishida, K. & Minoda, Y. (1979). Stability of indole-3-acetic acid to autoclaving, aeration and light illumination. *Agri. Biol. Chem.* 43:879-880.
- Yeoman, M.M., Kilpatrick, D.C., Miedzybrodzka, M. & Gould, A.R. (1978). Cellular interactions during graft formation in plants, a recognition phenomenon? *Symp. Soc. Exp. Biol.* 32:139-160.
- Yeoman, M.M. (1984). Cellular recognition systems in grafting. In *Encyclopedia of Plant Physiology*, Vol. 17, Cellular Interactions. H. F. Linskens & J. Heslop-Harrison (eds), pp. 453-472. Springer-Verlag Berlin-Heidelberg-New York-Tokyo.
- Yeoman, M.M. (1986) The present development and future of plant cell and tissue culture in agriculture, forestry, and horticulture. In *Plant Tissue Culture and Its Agricultural Applications*, L.A. Withers & P.G. Alderson (eds), pp.489-500. Butterworths, London.
- Yeung, F.C., Aitken, J., Biondi, S. & Thorpe, T.A. (1981). Shoot histogenesis in cotyledon explants of radiata pine. *Bot. Gaz.* 142: 494-501.
- Zaerr, J.B. & Mapes, M.O. (1982). Action of growth regulators. In *Tissue Culture in Forestry*, J.M. Bonga & D.J. Durzan (eds.), pp.231-255. Martinus Nijhoff/ Dr. W. Junk Publishers, The Hague.
- Zajackowski, S., Wodzicki, T.J. & Bruinsma, J. (1983). A possible mechanism for whole-plant morphogenesis. *Physiol. Plant.* 57: 306-310.
- Zobel, B. & Talbert, J. (1984). *Applied Forest Tree Improvement*. John Wiley & Sons. New York- Singapore.

In vitro adventitious shoot formation from embryonic and cotyledonary tissues of *Pinus brutia* Ten.

ANWAR A. ABDULLAH,* MICHAEL M. YEOMAN** and JOHN GRACE*

*Department of Forestry and Natural Resources;** Department of Botany; University of Edinburgh, Edinburgh EH9 3JU, Scotland, United Kingdom

(Received 16 October 1984; in revised form 14 May 1985; accepted 15 May 1985)

Key words: adventitious buds, Brutian pine, Calabrian pine, plant growth regulators

Abstract. Adventitious buds were induced when isolated whole embryos, and excised cotyledons from treated seeds of Calabrian pine (*Pinus brutia* Ten.) were cultured on a cytokinin-supplemented medium. The adventitious buds formed directly from the cotyledons. The highest number of bud primordia were formed, with both embryos and excised cotyledons, after 6 weeks on a BAP – supplemented Schenk and Hildebrandt medium under a 16 h photoperiod. The buds, when separated and maintained individually on a full-strength medium without growth regulators, developed into well-formed shoots within 4 weeks. The average number of harvested shoots obtained (> 1 cm in height) per seed over 24 weeks was 55; however, a maximum number of 152 shoots was obtained from one individual over the same period. The shoot forming capacity of the meristematic tissue was not lost after seven harvests.

Introduction

Tissue culture techniques provide an additional or alternative means of cloning forest trees, and may be used to achieve genetic improvement over a much shorter time than the traditional methods [10, 20, 22]. The potential use of tissue culture in forest improvement programmes has been described by a number of researchers [for example 6, 14, 22]. However, the application of tissue culture in forestry is still in its infancy, although economic, large scale micropropagation may not be far away [7]. Since gymnosperms constitute the major forest crop, special attention has been given to in vitro culture of species in this group over the last two decades. Most of the published papers have focussed on the effects of growth regulators on the morphogenetic response of explants. Recently, more attention has been given to the morphogenetic response of conifer tissue to specific macronutrients [8, 11].

Brutian or Calabrian pine (*Pinus brutia* Ten.) is native to the eastern Mediterranean Basin, and is highly prized because of its adaptation to both local climate and soil. It is extensively and successfully used in artificial afforestation [3, 12]. However, these plantations are still uneconomic due to lack of any ordered programme of tree improvement.

The aim of the present study was two-fold: (1) to develop a method for the induction of adventitious buds on two types of explant: excised whole

embryos, and excised cotyledons from treated seeds of Brutian pine, (2) to stimulate the development of these buds into shoots. This is, we believe, the first published account of the development of a suitable procedure for the *in vitro* culture of this species.

Material and methods

Plant material

Seeds collected in 1981 from plus trees of Calabrian pine (*Pinus brutia* Ten.) growing in northern Iraq, were used in these studies. Two types of explants were prepared: excised whole embryos (WE), and excised cotyledons from treated seeds (EC).

Dissection and culture of whole embryos

Calabrian pine seeds were disinfected in 10% (v/v) sodium hypochlorite solution for 15 min, washed under running tap water for 24 h, resterilized in 6% (v/v) 100 vol. hydrogen peroxide for 15 min, and rinsed three times with sterile distilled water. After stratification in the refrigerator for three days (4 °C), the seeds were resterilized in 6% (v/v) 100 vol. hydrogen peroxide for 5 min. Finally, the whole embryos were dissected out from the surrounding megagametophyte and cultured in Petri dishes containing agar medium. Explants were slightly inclined to the agar surface with the radicle submerged in the agar.

Dissection and culture of excised cotyledons

Seeds were sterilized as described above. To stimulate germination, the seeds were soaked for four days in 1% (v/v) 100 vol. hydrogen peroxide at 28 °C, rinsed five times in sterile distilled water and then cotyledons were excised from isolated whole embryos and planted horizontally on the agar medium in Petri dishes.

Culture media

To determine which medium would support the induction of adventitious buds in Calabrian pine, three culture media were tested, Schenk and Hildebrandt medium (SH) [25], Gresshoff and Doy medium (GD) [15], and Murashige and Skoog medium modified by Linsmaier and Skoog (LS) [19, page 119]. In all of the media tested minor elements were prepared according to Horgan and Aitken [16]. The pH of the medium for both SH and LS was adjusted with NaOH or HCl to 5.6–5.8, and 5.5 in GD medium before autoclaving at 15 psi, 121 °C for 20 min.

A factorial design was adopted to determine the optimum benzylamino-purine (BAP) concentration, with or without auxin, using two explant types, and with two photoperiods. Subsequently, an experiment was conducted to test the three media with the most effective BAP concentration.

Culture conditions

Both types of explant were cultured in 90 × 15 mm Petri dishes (2–5 explants per dish) containing 20 ml of (SH, GD, or LS) medium. The plates were sealed with Parafilm[®], and incubated in a growth room at 23 ± 1 °C (26 ± 1 °C inside the plates) using a photon flux of 80 μmol m⁻² s⁻¹ (400–700 nm). The light source consisted of a bank of 6 white fluorescent tubes (85 W), interspersed with 3 60 W incandescent lamps. All the experiments were conducted under two photoperiods (16 and 24 h), and the plates were set out in a randomized block design.

Induction of adventitious buds

Adventitious bud induction was achieved when both types of explant were maintained for 6 weeks on media supplemented with growth regulators. Cytokinin (CK) and auxin in the form of BAP and NAA were applied either singly or in combination of concentrations of 1, 3, 5, 7 mg l⁻¹, and 0.01 mg l⁻¹ respectively. A higher concentration of 10 mg l⁻¹ BAP was also tested with LS medium.

Adventitious shoot formation and in vitro multiplication

After 6 weeks on medium supplied with growth regulators, buds 1–3 mm were transferred individually into 100 or 200 ml conical flasks containing 25 or 50 ml respectively on medium without growth regulators. Every fourth week, shoots (10–20 mm in height) were harvested, counted, and subcultured into rooting medium, while the remaining meristematic tissue was transferred again onto fresh medium devoid of growth regulators to produce more shoots. In order to increase shoot formation as well as shoot multiplication, SH medium at half and full strength was tested. In one experiment the terminal portions of the individual shoots were excised with both the shoots and their bases placed back on SH medium.

A comparison of shoots placed on GD medium with 2% versus 1% sucrose was also made. Buds were also subcultured either individually or as a cluster attached to the explant on LS medium.

Anatomical studies

Tissue for anatomical observations was fixed in a buffered mixture (pH = 7.2) of 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde, dehydrated in an ethanol – tertiary butanol series, embedded in paraffin, sectioned as 10 μm slices, and stained in tannic acid-ferric chloride – safranin [17, 18].

Results

(1) Bud induction

Influence of plant growth regulators. Both types of explant responded within one week by swelling and greening on BAP medium; the hypocotyls

sometimes became reddish green or dark green. The radicles did not show a response either in colour or in cell proliferation. A maximum number of 8 cotyledons was observed on each explant. In both types of explant, two different responses were observed depending on whether the cotyledons, within each cultured explant, were in contact with the medium or not. Cotyledons in contact with the medium proliferated rapidly, producing callus, and after 6 weeks a mass of tiny buds each with a cluster of small scale leaves appeared on the tissue (Figure 1). However, cotyledons not in contact with the medium showed less swelling, and only one bud was induced at each cotyledon tip within 4–5 weeks (Figure 2). Occasionally, with both types of explant, cotyledons swelled to a limited extent, and numerous clusters of buds arose at cotyledonary axils within 3–4 weeks (Figure 3). Subsequently these buds developed quickly into normal shoots.

Histological observations were made of cotyledons at various stages of regeneration. After 3 weeks of culture, some densely cytoplasmic cells, in the epidermal layers underwent division, and gave rise to meristematic centres. Subsequently, these developed into bud primordia (Figure 4). However, a defined form of adventitious bud was observed after 5–6 weeks (Figure 5).

The maximum morphogenetic response was observed on BAP medium after 6 weeks. Explants were then transferred onto medium without growth regulators, or the buds quickly became overgrown with callus. The percentage of explants forming buds was affected by the concentration of added growth substances, a higher percentage occurring on the lower level of BAP [$1, 3 \text{ mg l}^{-1}$] (Figure 7)]. The percentage of buds associated with the cotyledonary axils was often low, and this was not affected significantly by the level of cytokinin. The number of buds per explant tended to increase as the level of BAP was raised; however, the effect of BAP concentration on the number of buds per explant was not significant. The mean number of induced buds at the cotyledonary axils, and those induced on cotyledonary callus and tips ranged between 6–30, and 1–8 respectively.

When auxin (NAA) was added at 0.01 mg l^{-1} to GD medium containing BAP at a range of concentrations bud induction increased slightly (30 to 34%). However, this increase was not statistically significant at any level. Moreover, the presence of auxin in the medium even at an extremely low level promoted excess callus proliferation.

Influence of explant sources. Table 1 shows that there are some differences (not statistically significant) between the differentiation of both type of explants. However, excised cotyledons tended to differentiate more on SH and GD media.

Influence of photoperiod. Morphogenetic response of both explant types cultured on SH and GD media were promoted, perhaps surprisingly, in a

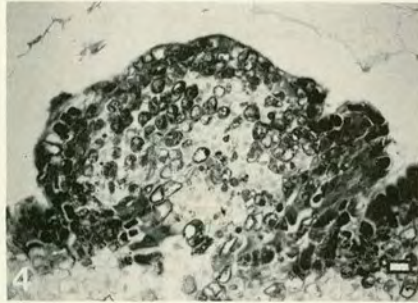
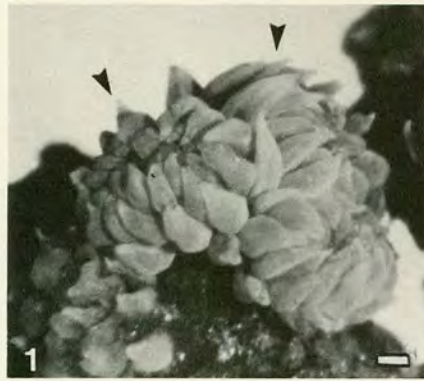


Figure 1. Adventitious buds (arrows) induced on the cotyledonary callus after 6 weeks in culture.

Figure 2. Cotyledon tips with adventitious buds (arrows) after 5 weeks in culture.

Figure 3. Bud development (arrows) at the axils of cotyledons of *Pinus brutia* after 4 weeks on medium containing 3 mg l^{-1} BAP.

Figure 4. Early stage of adventitious bud development showing an apical meristem flanked by a pair of needle primordia after 4 weeks in culture.

Figure 5. Late stage of adventitious bud development after 6 weeks in culture.

Figure 6. Normal shoot formation from induced buds on cotyledons. 4 weeks after subculturing from medium containing BAP onto medium without growth regulators.

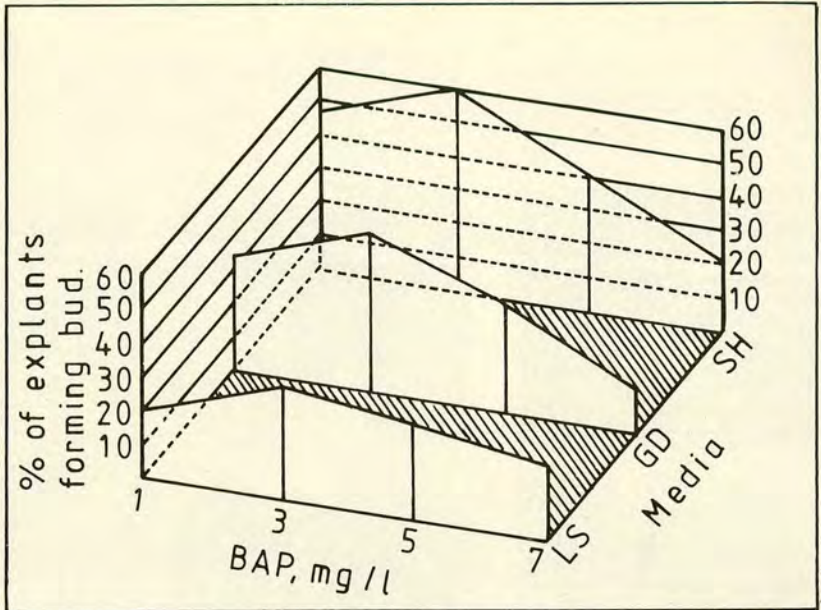


Figure 7. Effect of various concentrations of BAP and three types of medium on the frequency of bud formation on explants (both excised cotyledons and excised whole embryos) of *Pinus brutia* Ten. 42–62 explants were tested at each concentration of BAP with each medium.

Table 1. Mean number of Calabrian pine explants forming bud using two sources of explant (excised whole embryos and excised cotyledons from treated seeds) after 6 weeks on various media supplemented with BAP

Media	Total no of cultured explants	Mean number* of explants forming buds	
		Explants sources	
		Whole embryos	Excised cotyledons
SH	128	45a	58a
GD	128	37b	42b
LS	105	29c	25c

*Means within each medium followed by the same letters are not significantly different at any level (chi-squared test).

16 h photoperiod where the mean number of explants forming buds under a 16 h photoperiod or in continuous illumination was 79 versus 15 and 52 versus 27 on SH and GD media respectively. Moreover, cultured explants maintained under continuous illumination were dominated by callus growth, and most of these turned red and died.

Influence of media. Best results in regard to the frequency of explant forming buds with all levels of BAP applied were achieved with SH medium, while

Table 2. Mean number of shoots produced per explant (both excised embryos and excised cotyledons from treated seeds) of Calabrian pine over 24 weeks on SH and GD media devoid of growth substances. These shoots developed from induced buds at various levels of BAP. Only shoots > 10 mm in height were considered [+] good response, [(+)] slight response, [-] poor response.

Media	BAP levels mg/l used for induction	Response for shoot formation	Mean number* of shoot/explant over 24 weeks		
			Max	Min	Mean
SH	1	+	71	12	35.4a
	3	+	152	29	55.3a
	5	-	20	10	13.2b
	7	-	20	11	15.2b
GD	1	(+)	36	10	25.3a
	3	+	51	21	28.5a
	5	-	15	6	11.3b
	7	-	26	13	16.3b

*Means within SH medium followed by different letters are different at 1%, and those within GD medium different at 5% level (chi-squared test).

these responses with all BAP concentrations declined considerably on LS medium. (Figure 7).

(II) Bud elongation and shoot formation

The capacity to produce shoots varied considerably between the explants, and was also affected by the initial BAP levels used for bud induction (Table 2). Shoot formation was also influenced by the type of buds; both buds associated at cotyledonary axils, and those which arose on the cotyledon tips showed rapid development into normal shoots (Figure 6). Those adventitious buds which differentiated on the callus in contact with the agar did not elongate further. However, the best shoot formation occurred with those buds induced at the lower levels of BAP (1, 3 mg l⁻¹) on both SH and GD media (Table 2).

Of the various techniques employed for bud elongation and shoot multiplication, the best results were achieved by subculturing individual buds, which formed after 6 weeks incubation on SH + BAP (3 mg l⁻¹) into full-strength SH medium lacking growth substances. After 4 weeks, individuals which had grown into normal shoots were harvested, and the remaining material was subcultured into fresh medium of the same composition. No real differences on shoot formation could be obtained with 1% or 2% sucrose on GD medium. However, a remarkable enhancement was noticed with bud elongation on LS medium when the buds were subcultured individually rather than transferred unseparated. Individually subcultured buds grew more vigorously and uniformly compared with unseparated buds where only one or two individuals elongated and dominated the rest. It was also observed that the procedure of excising the shoots was ineffective in stimulating shoot multiplication.

A maximum of 152 shoots per seed with an average of 55 shoots has been obtained over a period of 24 weeks. Only shoots more than 10 mm in height were taken into consideration.

After the sixth harvest some of the shoots which elongated on SH medium turned yellow. Occasionally, abnormal shoots also appeared on both SH and GD media, the shoots turned bright green and their needles adhered to each other.

Discussion

From the work of others [10, 20], it is clear that conditions necessary to promote organogenesis in cultured tissue from conifers must be developed for each individual species. Data presented in this study reveal that cytokinin (BAP) supplied at a low level to the culture medium elicits a maximum response in terms of bud induction on embryonic tissues of *Pinus brutia* Ten. Similar observations have been reported with other gymnosperm species [11, 27]. Our results also indicate that cytokinin alone is sufficient to bring about shoot formation, and this has been confirmed with several other conifer species [5, 26]. As observed with other species, exogenous auxin supports more callus production than organogenesis [8]. Indeed this effect has also been recorded with cultured needles of the species used in this study [Abdullah, unpublished results]. As it has been shown that the developmental state of the cotyledons at the time of excision has a critical influence on the subsequent stages of organogenesis [1, 28], the statistically insignificant increase achieved so far with excised cotyledons must be taken into consideration when any attempt is made to optimize this procedure.

It is also evident from these results that a 16 h photoperiod supports more bud initiation than continuous illumination, and this is consistent with the fact that most other workers have used a photoperiodic regime simulating day and night, rather than continuous day [for example 1, 24].

Among the three media employed in this study, the promotion of organogenesis was best on SH medium. This medium is characterized by a high concentration of potassium, and the beneficial effects of this element on organogenesis have already been recognized [9]. Moreover, the level of calcium and its ratio to other macroelements is also considered to be important [8]. In SH medium the concentration of calcium is intermediate between that in LS and GD media [15, 19, 25]. In fact this medium has been found to be very useful by other authors [23, 24]. According to Amos and McCown (2), and David and David (9) BAP at a low level stimulates shoot production, while a higher concentration inhibits shoot formation [2, 11]; this fact is confirmed with *Pinus brutia*. The yellow shoots occasionally produced on SH medium in this study have also been observed with *Pinus radiata* [16]. Although, so far in studies conducted here in Edinburgh this problem has been overcome by an increase in the iron content of SH medium

to the level used in LS medium [Abdullah, unpublished results]. The abnormal shoots grown in our system have also been reported in *Picea abies* [4], and in *Pinus radiata* [1]. However, it has been found that such problems can be controlled by altering the agar concentration of the culture medium [13].

Under the best conditions employed in this study, shoot production of up to 152 per clone with an average of 55 shoots per seed was obtained over 24 weeks. Even then the meristematic tissue mass was still healthy and was presumably capable of producing more shoots, however, so far this aspect has not been investigated. The average of shoot production obtained here is equivalent of the highest levels from conifer embryonic tissue in vitro, where an average of 9 shoots per embryo with a wide range of 0–248 shoots per embryo has been recorded within 15 weeks in *Pinus radiata* [16]. Also in *Pinus taeda*, 16 shoots per embryo as an average and up to more than 100 shoots per clone has been achieved within 18–26 weeks [21].

Rooting of the shoots produced was achieved on an appropriate combination of growth substances. Details concerning development, survival, and establishment of regenerated plantlets will be reported in a later publication.

References

1. Aitken J, Horgan KJ, Thorpe TA (1981) Influence of explant selection on the shoot forming capacity of juvenile tissue of *Pinus radiata*. *Can J For Res* 11:112–117
2. Amos RR, McCown BH (1981) Micropropagation of member of coniferae. *J Hort Sci* 16(3):453
3. Anders O (1969) Forest tree in Iraq. Ninevah, Iraq: Mosul University press, pp 7–10
4. Arnold VS (1982) Vegetative propagation of *Picea abies*. In: Fujiwara A (ed) Proceedings of the 5th international congress of tissue and cell culture. Tokyo, Japan: Maruzen Co. Ltd, pp 753–754
5. Biondi S, Thorpe TA (1982) Clonal propagation of forest tree species. In: Rao AN (ed) Proceedings COSTED Symposium on tissue culture of economically important plants. Singapore: National university of Singapore, pp 197–204
6. Bonga JM (1977) Application of tissue culture in forestry. In: Reinert J, Bajaj YPS (eds) Plant cell tissue, and organ culture. Berlin: Springer-Verlag, pp 93–108
7. Bonga JM, Durzan DJ (1982) Introduction. In: Bonga JM, Durzan DJ (eds) Tissue culture in forestry. Boston, London: Dr W Junk, pp 1–3
8. Bornman CH (1983) Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies* in vitro. *Physiol Plant* 57:5–16
9. David HK, Isemukali K, David A (1978) Obtention de plants de pin maritime (*Pinus pinaster* Sol.) à partir de brachyblastes ou d'apex caulinaires de très jeunes sujets cultivés in vitro. *CR Acad Sc* 287:245–248
10. David A (1982) In Vitro Propagation of gymnosperms. In: Bonga JM, Durzan DJ (eds) Tissue culture in forestry Boston, London: Dr W Junk, pp 72–108
11. David A, David H, Mateille T (1982) In vitro adventitious budding on *Pinus pinaster* cotyledons and needles *Physiol Plant* 56:102–107
12. Davis PH (1965) Flora of Turkey, vol. 1. Edingburgh: Aldin Publishing Company, p 74
13. Debergh P, Harbaoui Y, Lemeur R (1981) Mass propagation of globe artichoke

- (*Cynara scolymus*): evaluation of different hypotheses to overcome vitrification with special references to water potential. *Physiol Plant* 53:181-187
14. Durzan DJ (1982) Cell and tissue culture in forest industry. In: Bonga JM, Durzan DJ (eds) *Tissue culture in forestry*. Boston, London. Dr W Junk: pp 36-71
 15. Gresshoff PM, Doy CH (1972) Development and differentiation of haploid *Lycopersicon esculentum* (tomato) *Planta (Berl.)* 107:161-170
 16. Horgan K, Aitken J (1981) Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. *Physiol Plant* 53:170-175
 17. Jenson WA (1962) *Botanical Histochemistry*. San Francisco and London: WH Freeman and company
 18. Karnovsky MJ (1965) A Formaldehyde-Glutaraldehyde fixative of high osmolality for use in electron microscopy. *J Cell Biol.* 27:137A-138A
 19. Linsmaier EM, and Skoog F (1965) Organic growth factor requirements of tobacco tissue culture. *Physiol Plant* 18:100-127
 20. Mott RL (1981) Tree. In: Conger BV (ed) *Cloning agricultural plants via in vitro techniques*. Boca Raton Florida: CRC Press, pp 217-254
 21. Mott RL, Amerson HV (1981) A tissue culture process for the clonal production of Loblolly pine plantlets. *NC Agric Res Serv Tech Bull.* 271:1-14
 22. Rao AN, Lee SK (1982) Importance of tissue culture in tree improvement. In: Fujiwara A. (ed) *Proceedings of the 5th international congress of tissue culture*. Tokyo, Japan: Maruzen Co., Ltd. pp 715-718
 23. Reilly K, Washer J (1977) Vegetative propagation of radiata pine by tissue culture: Plantlet formation from embryonic tissue. *N Z J For Sci* 7(2):199-206
 24. Rumary C, Thorpe TA (1984) Plantlet formation in black and white spruce. I. in vitro techniques. *Can J For Res* 14:10-16
 25. Schenk RU, Hildebrandt AC (1972) Media and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell culture. *Can J Bot* 50:199-204
 26. Thorpe TA (1980) Organogenesis in vitro, structural, physiological and biochemical aspects. In: Vasil IK (ed) *International review of cytology*. Suppl 11A. London: Academic Press, pp 71-111
 27. Webb KJ, Street HE (1977) Morphogenesis in vitro of pinus and picea. *Acta Horticulture* 78:259-269
 28. Yeung EC, Aitken J, Biondi S, Thorpe TA (1981) Shoot histogenesis in cotyledon explants of radiata pine. *Bot Gaz* 142(4):494-501



**INTERNATIONAL
SYMPOSIUM
PLANT TISSUE
AND CELL CULTURE
APPLICATION TO CROP
IMPROVEMENT
OLOMOUC
CZECHOSLOVAKIA
24-29 SEPTEMBER
1984**

PROCEEDINGS

**EDITED BY F. J. NOVÁK, L. HAVEL, J. DOLEŽEL
INSTITUTE OF EXPERIMENTAL BOTANY**

**CZECHOSLOVAK ACADEMY
OF SCIENCES
PRAGUE 1984**

IN VITRO ORGANOGENESIS ON PINUS BRUTIA NEEDLES AND SHOOT TIPS

ANWAR A ABDULLAH*, MICHAEL M YEOMAN**, JOHN GRACE*

*Department of Forestry and Natural Resources, Edinburgh University,
Edinburgh, EH9 3JU

**Department of Botany, Edinburgh University, Edinburgh, EH9 3JH

Introduction

During recent years, important advances have been made in developing methods for in vitro propagation of trees (MOTT 1981). However, the application of tissue culture in forestry is still in its infancy, although, economic, large scale micropropagation may not be far away (BONGA and DURZAN 1982), Calabrian pine (*Pinus brutia* Ten.) is an important forest tree species limited to the eastern Mediterranean countries. However, no organised programme for tree improvement has yet been established for this species. Towards this end, we have been developing methods for organ culture of Calabrian pine, in order ultimately to achieve a practical means of vegetative propagation and cloning of this species. Results have been encouraging. Undoubtedly, needle and shoot tip culture offer a great potential to obtain truly clonal lines. A procedure for plantlet regeneration, through four stages, could be successfully developed using excised needles and shoot tips as an initial plant material.

Materials and Methods

Seedlings (8 week-old) were first disinfected in 7 % NaOCl solution for 7 min, followed by immersion for 4 min in 6% 100 vol. H₂O₂, and then rinsed 4 times in sterile distilled water. Needles (10-15 mm) were gently removed with forceps from the upper 1 cm region of the shoot tip, and batches of 10 needles were cultured in 50 mm Petri dishes containing SCHENK and HILDEBRANDT medium modified as follows: macronutrients of SH medium (1972), micronutrients as prepared by HORGAN and AITKEN (1981), iron salts of MURASHIGE and SKOOG medium (1962). SH medium was applied either as solution or solidified with 0.6% Bacto agar. In another study, shoot tips 10-25 mm long were sterilized as above, then isolated from the surrounding needles, and cultured either with or without their apical bud in Petri dishes containing SH medium as described above. The applied media were supplied with various cytokinin types and concentrations, with or without auxin. A mixture of two cytokinins was also tested for both the procedures.

Induction of Bud, Shoot, and the Formation of Plantlets

The initiation of adventitious buds on excised needles, and axillary buds from excised shoot tips were considered in terms of various factors involved such as cytokinin types (BAP, Kn, 2ip) and levels (0, 1, 3, 5, 10 mg l⁻¹), and the state of the applied media. The requirement of bud development and shoot formation from induced buds on excised needles, or from developed axillary buds on excised shoot tips were examined. The inclusion of activated charcoal in the applied medium in the former case, and exclusion of growth substances in the medium in the latter case were investigated. Rooting of induced shoots was tested on different combinations of two auxins (NAA and IBA) with or without a low level of BAP.

Results and Discussion

Exogenous cytokinin was essential for the induction of the adventitious bud on cultured needles, however both exogenous cytokinin and removal of apical bud promoted axillary bud formation on excised shoot tips. BAP gave the highest yield of adventitious and axillary buds, while 2ip was the least effective cytokinin. A similar result was reported by ARNOLD (1982). The highest percentage of needle-forming buds was obtained on SH medium containing $5-10 \text{ mg l}^{-1}$ BAP within 8-10 weeks, and the most effective level for highest yield of axillary bud on excised shoot tip was 3 mg l^{-1} BAP. However, a mixture of BAP and Kn (1 mg l^{-1} for each) was more effective in this respect than the case with one cytokinin for both procedures. This effect was emphasized by CHENG (1977), and ARNOLD (1982). Best results in terms of higher percentage of explant forming bud was achieved on agar SH medium, whereas, this frequency declined when explants were cultured in liquid SH medium.

In both procedures, bud multiplication of 2-3 fold could be achieved within 4-6 weeks when induced buds were incubated on SH medium supplemented with a mixture of BAP+Kn (1 mg l^{-1}).

Normal shoots ($>10 \text{ mm}$ in height) could be obtained for both procedures within 4-6 weeks on medium which included 1% activated charcoal in the former procedure, and on a medium without growth substances in the latter procedure. Results of a series of experiments on rooting of induced shoots, for both procedures, demonstrated that shoots had to be subjected to a combination of two auxins with a low concentration of cytokinin, with subsequent transfer onto a half-strength medium devoid of growth substances. Rooting percentage of up to 73 could be obtained within 4-8 weeks on such a medium. Regenerated plantlets could undergo a normal autotrophic growth when transferred to greenhouse conditions.

References

- ARNOLD, V.S., (1982): In A.Fujiwara (Ed.). Proceedings Of The 5th International Congress of Tissue and Cell Culture. Maruzen Co.Ltd., Japan, pp 753-754.
- BONGA, J.M., DURZAN, D.J. (1982): In J.M.BONGA and D.J.DURZAN (Eds.). Tissue Culture in Forestry. Dr. W.Junk, Boston, London, pp 1-3.
- CHENG, T.Y. (1977): Plant Sci. Lett. 9:179-187.
- HORGMAN, K., AITKEN, J. (1981): Physiol. Plant. 53: 170-175.
- MOTT, R.L. (1981): In B. V. CONGER (Ed.). Cloning Agricultural Plants via In Vitro Techniques. CRC Press, Boca Raton Florida, pp 217-254.
- SCHENK, R.U., HILDEBRANDT, A.C. (1972): Can. J. Bot. 199-204.

Rapid micropropagation of Calabrian pine from primary and secondary buds on shoot explants

ANWAR A. ABDULLAH AND JOHN GRACE

Department of Forestry and Natural Resources, University of Edinburgh, Edinburgh, Scotland EH9 3JU

AND

MICHAEL M. YEOMAN

Department of Botany, University of Edinburgh, Edinburgh, Scotland EH9 3JH

Received September 30, 1985¹

Accepted January 23, 1986

ABDULLAH, A. A., J. GRACE, and M. M. YEOMAN. 1986. Rapid micropropagation of Calabrian pine from primary and secondary buds on shoot explants. *Can. J. For. Res.* **16**: 637–641.

Axillary shoot production was achieved in 6 weeks using excised shoot explants of *Pinus brutia* Ten. on a modified Schenk and Hildebrandt medium containing cytokinin. Primary shoots arose from existing axillary buds and secondary buds arose from bases of the primary shoots. Their production could be increased by regulating the cytokinin level and by surgical removal of apical buds from the cultured explants. However, the best performance was achieved with a low level of 6-benzylaminopurine (3×10^{-6} M) or a mixture of 6-benzylaminopurine and kinetin (10^{-6} M of each). Subsequent transfer to a cytokinin-free medium resulted on average in the production of 43 shoots per cultured explant and up to 67 shoots per clone within 12 weeks. When the primary shoots, which had already produced one crop of secondary shoots, were maintained under conditions favourable for shoot production, a doubling in number was obtained within 4–6 weeks. To encourage further elongation, newly formed shoots were incubated for 2 weeks on a cytokinin-free medium to which 1% activated charcoal was added. The time taken with this method was much shorter than with other published methods and is, therefore, likely to be important for the vegetative propagation and multiplication of selected seedlings of this species.

ABDULLAH, A. A., J. GRACE et M. M. YEOMAN. 1986. Rapid micropropagation of Calabrian pine from primary and secondary buds on shoot explants. *Can. J. For. Res.* **16**: 637–641.

La production de tiges axillaires peut être obtenue en 6 semaines en cultivant des explants caulinaires excisés de *Pinus brutia* Ten. sur milieu de Schenk et Hildebrandt contenant de la cytokinine. Des tiges primaires se développent à partir des bourgeons axillaires déjà existants, et des bourgeons secondaires apparaissent à partir de la base des tiges primaires. Leur production peut être augmentée en ajustant la teneur en cytokinine ou par ablation des bourgeons apicaux sur les explants en culture. Tout de même, la meilleure performance s'obtient avec une faible teneur en benzyl-6 aminopurine (3×10^{-6} M) ou avec un mélange de celle-ci avec de la kinétine (10^{-6} M de chacune). Suite à un transfert dans un milieu sans cytokinine, les auteurs ont obtenu une production moyenne de 43 tiges par explant et jusqu'à 67 tiges par clone, en moins de 12 semaines. Lorsque les tiges primaires qui ont déjà produit une récolte de tiges secondaires, sont maintenues dans des conditions qui favorisent la production des tiges, il est possible de doubler ce nombre en moins de 4–6 semaines. Pour encourager une élévation plus poussée, on incube les tiges nouvellement formées pendant 2 semaines sur un milieu sans cytokinine, additionné de 1% de charbon de bois activé. Cette méthode requiert beaucoup moins de temps que celles déjà publiées et conséquemment elle pourrait s'avérer importante pour la propagation végétative et la multiplication de plantules sélectionnées de cette espèce.

[Traduit par la revue]

Introduction

Plant regeneration through the development of existing buds permits the establishment of a genetically homogeneous clonal population (David 1982). So far, this approach has proved difficult with conifers, except with *Pseudotsuga menziesii* (Boulay 1976), *Abies balsamea* (Bonga 1977), *Pinus pinaster* (David et al. 1978), and *Pinus nigra* (Jelaska et al. 1981). However, unlike most other conifers, juvenile pines normally bear an axillary bud at each scale leaf on the shoot axis and most of these axillary buds can develop into needle-bearing short shoots (Borthwick 1899; Stone and Stone 1943; Fink 1984).

Calabrian pine (*Pinus brutia* Ten.) is native to the eastern Mediterranean Basin. It is preferred because of its ability to grow in diverse climates and on various soil types, which, together with its good form, is the reason it is used extensively in afforestation and reforestation of the region (Anders and Kettaneh 1969; Kayacik and Yaltiric 1970). In this study, the potential of existing axillary buds as a source of material for the *in vitro* multiplication of seedling material of *Pinus brutia* has

been evaluated, as well as some of the factors involved in bud development, multiplication, and elongation.

Materials and methods

Plant material

Seeds were collected from a single plus tree (No. 45, Zawita, Iraq) of *Pinus brutia*. They were germinated, after stratification in the refrigerator for 2 weeks, and soaked for 24 h in tap water. The seeds were sown in a mixture of 1:1 sand-peat (granulated sphagnum moss peat, pH 4.0) in the greenhouse (18 h light : 6 h dark photoperiod; 20:16°C, day:night). Seedlings (10 weeks old) were surface sterilized by immersion for 7 min in a solution of sodium hypochlorite (7%, v/v), resterilized in 6% 100 vol. hydrogen peroxide for 4 min, and rinsed four times in sterile distilled water. Shoots 20–25 mm long were excised from the seedlings and all the needles were carefully removed. These shoots were then cultured either as (A) shoot explants without apical buds or (B) entire shoot explants with apical buds. In another study, explants from 6-month-old seedlings were cultured in the same way.

Culture conditions

All the explants were maintained on Schenk and Hildebrandt (SH) medium modified as follows (milligrams per litre): KNO₃, 2500; MgSO₄·7H₂O, 400; NH₄H₂PO₄, 300; CaCl₂·2H₂O, 200; MnSO₄·

¹Revised manuscript received January 15, 1986.

TABLE 1. Effect of two levels of 6-benzylaminopurine (BAP) and a mixture of two cytokinins (BAP and kinetin (Kin)) on the production of primary axillary shoots and secondary buds on cultured shoot explants of *Pinus brutia* Ten. after 6 weeks on Schenk and Hildebrandt medium

Cytokinin concn. (M)	Mean no. of primary shoots per explant	Mean height (mm) of primary shoot	Mean no. of secondary buds per explant
3×10^{-6} BAP	2.3±0.1a	7.8±0.3a	6.5±0.5a
5×10^{-6} BAP	1.0±0.09b	4.3±0.4b	4.5±0.4b
10^{-6} BAP + 10^{-6} Kin	2.3±0.1a	8.8±0.3a	4.6±0.3b
CV	35	37	51

NOTE: Means in columns not followed by a common letter are significantly different ($P = 0.01$, Duncan's multiple-range test). CV, coefficient of variation.

4H₂O, 20; H₃BO₃, 5; ZnSO₄·7H₂O, 1; KI, 1; CuSO₄·5H₂O, 0.2; NaMoO₄·2H₂O, 0.2; CoCl₂·6H₂O, 0.2; thiamine HCl, 5; nicotinic acid, 5; pyridoxine HCl, 0.5; *myo*-inositol, 1000; FeSO₄·7H₂O, 27.8; NaEDTA, 37.3 (Abdullah *et al.* 1985). The modified SH medium was solidified with 0.6% Bacto-agar and the pH was adjusted to 5.6–5.8 before autoclaving for 20 min at 121°C. Explants were cultured in 90 × 15 mm Petri dish (three or four explants per dish) containing 20 mL of the modified SH medium. The plates were sealed with Parafilm® and maintained in a growth chamber with a 16-h photoperiod (fluorescent tubes 85 W, and incandescent lamps 60 W with a photon flux density of 80 μmol m⁻² s⁻¹ (400–700 nm), and a temperature of 23 ± 1°C. The cultures were subcultured onto a fresh medium at 4 week intervals.

Shoot formation from primary axillary buds

To induce the growth of the primary axillary buds, explants (with or without an apical bud) were incubated initially for 6 weeks on the modified SH medium supplemented with 3% sucrose and either 6-benzylaminopurine (BAP) alone at a concentration of 3 or 5 × 10⁻⁶ M or together with kinetin (Kin), each at 10⁻⁶ M.

The shoots that developed from primary axillary buds on cultured explants were evaluated for the number produced and their length after 6 weeks on the cytokinin-containing medium.

Shoot formation from secondary buds

During culture, many secondary buds were also observed at the base of the newly formed primary shoots. Following evaluation (number and length), these shoots were subcultured onto a half-strength medium without cytokinin, but containing 2% sucrose. The elongation of induced secondary buds was also tested on the same medium, but with 1% activated charcoal added.

Shoot multiplication

Two techniques were used for shoot multiplication. First, secondary buds were transferred every 4 weeks onto a medium without cytokinin and, after 12 weeks, the number and length of the shoots formed was determined. Second, individual primary shoots, which had already produced a crop of secondary shoots, were maintained on a medium with BAP + Kin (10⁻⁶ M for each) for 4–6 weeks. The second crop of buds was subcultured onto SH medium supplemented with 1% activated charcoal for 2 weeks to encourage elongation.

Statistical analysis

An analysis of variance was performed on the data from the factorial

(two factors; level of cytokinin and shoot explants with or without an apical bud) experiments. Statistical evaluation also included calculation of the estimated standard error (SE) and coefficient of variation (CV). Least significant range (LSR) was calculated using Duncan's multiple-range test. The number of explants used for each treatment ranged from 60 to 120 and all the experiments were conducted twice.

Anatomical studies

Specimens for anatomical study were fixed in Karnovsky's fixative (Karnovsky 1965) at 4°C for 24 h for light microscopy (LM) and for 36 h for scanning electron microscopy (SEM). Tissues for both SEM and LM received a postfixation treatment in 1% aqueous osmium tetroxide solution for 2–3 h. Specimens for LM were dehydrated in an ethanol series, followed by propylene oxide, and embedded in Epon (Roland 1978). Sections were cut on a Reichert–Jung Ultracut microtome at a thickness of 0.5 μm. All the sections were stained with 0.5% toluidine blue in 1% Borax. Specimens for SEM were dehydrated in an acetone series and critical-point dried under liquid CO₂ (Roland 1978).

Results

It can be seen from Fig. 1 that culture induced development of the existing axillary buds on the shoot axis. From the anatomical and SEM observations, it can also be observed that at explantation, these buds were at an early stage of development (Figs. 2 and 3) and grew out within 1 week on an SH medium containing cytokinin (Figs. 4 and 5). Only another 1–2 weeks was required to complete development into vigorous buds (Fig. 6). Further development of these buds into normal shoots was associated with the appearance of clusters of secondary buds at the bases of the primary buds (Fig. 7) after a total incubation time of 6 weeks on the same medium. In general, the frequency of explants that formed buds was 98–100%. However, yields of buds and shoots per explant and their appearance varied markedly according to the treatments applied (Tables 1 and 2).

Shoot formation from primary axillary buds

After 6 weeks on the shoot-forming medium (with cytokinin), the maximum number of vigorous shoots produced per

FIG. 1. Calabrian pine seedling with an axillary bud (arrow) (explant before culture). Scale bar = 0.5 mm. FIG. 2. Scanning electron micrograph of the same bud as in Fig. 1 showing the bud enclosed within bud scales (arrow). Scale bar = 100 μm. FIG. 3. Median longitudinal section of the same bud as in Fig. 1 showing typical features of a bud at an early stage with leaf primordia (black arrow) and bud scales (open arrow). Scale bar = 1 mm. FIG. 4. Scanning electron micrograph of the same structure after 1 week on a medium with cytokinins, showing how the bud scales have started to open (arrows). Scale bar = 100 μm. FIG. 5. Median longitudinal section of the same structure as in Fig. 4 showing a developed primordium. Scale bar = 1 mm. FIG. 6. Median longitudinal section of well-developed bud after 2–3 weeks on the culture medium. Scale bar = 1.5 mm. FIG. 7. Primary axillary shoot formation (black arrows) with masses of secondary buds (white arrows) on cultured explants after 6 weeks on cytokinin medium. Scale bar = 2.5 cm. FIG. 8. Production of primary axillary shoots (arrows) on two types of cultured explants (a) shoot pieces without an apical bud; (b) shoot pieces with an apical bud. Scale bar = 2 cm.

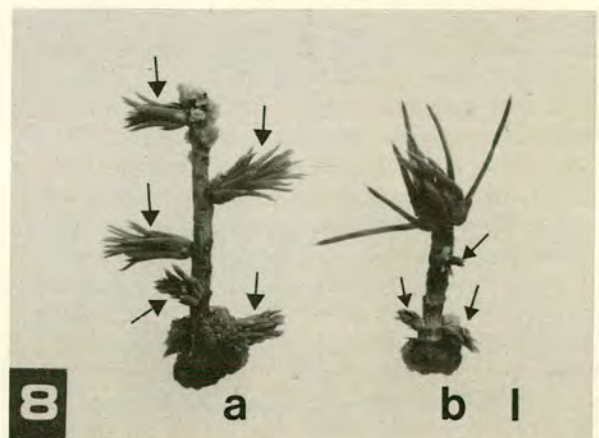
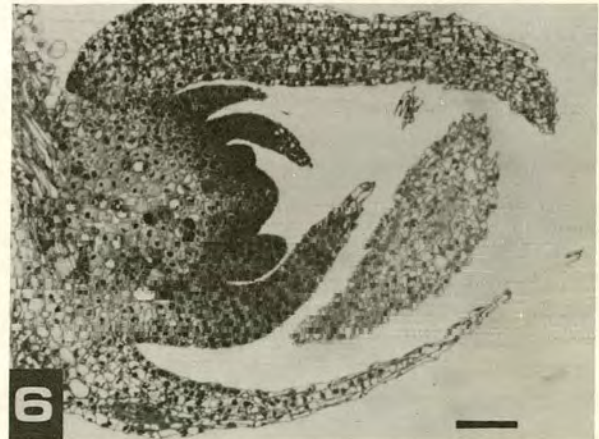
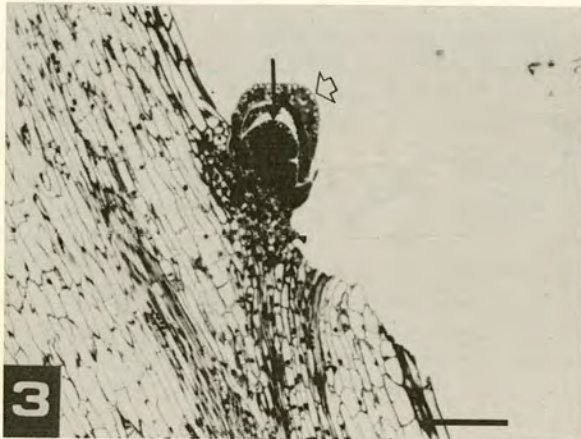
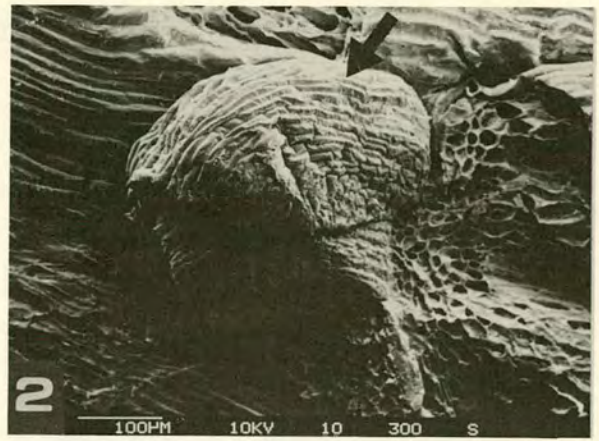
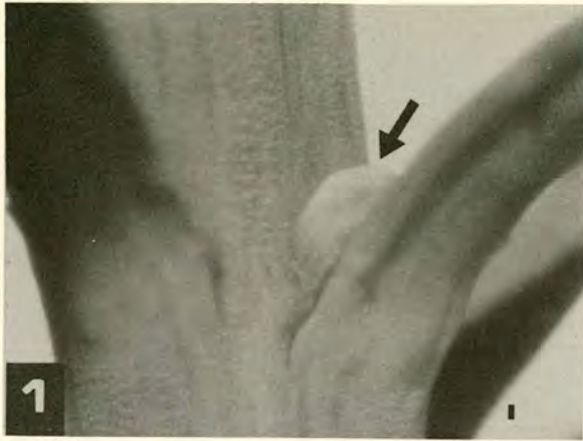


TABLE 2. Effect of excision of apical bud from cultured shoot explants of *Pinus brutia* Ten. on the production of primary axillary shoots and secondary buds after 6 weeks on Schenk and Hildebrandt medium supplement with cytokinin

Types of cultured explants	Mean no. of primary shoots per explant	Mean height (mm) of primary shoot	Mean no. of secondary buds per explant
Shoot without apical bud	2.3±0.1a	7.6±0.3a	7.2±0.3a
Shoot with apical bud	1.4±0.1b	6.4±0.4b	3.2±0.1b
CV	35	37	51

NOTE: Means in columns not followed by a common letter are significantly different ($P = 0.01$). CV, coefficient of variation.

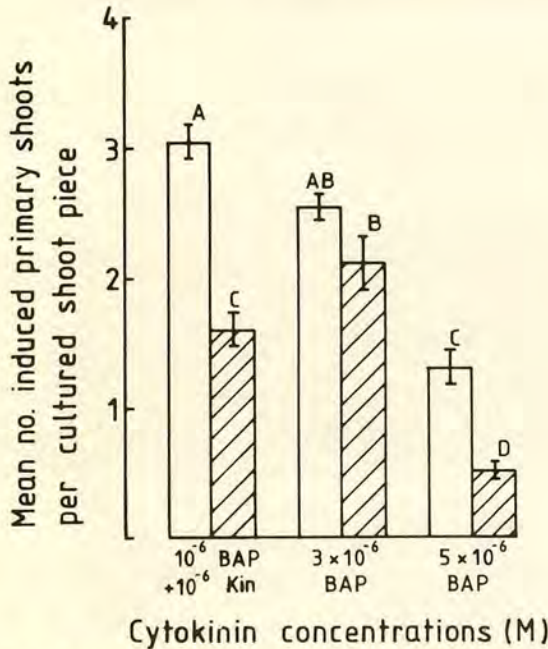


FIG. 9. Interaction of applied cytokinin levels and excision of apical bud from the cultured shoots on yield of primary shoots on cultured shoot explants of *Pinus brutia* Ten. after 6 weeks on Schenk and Hildebrandt medium: □, without apical bud; ▨, with apical bud. Vertical bar represents the standard error. Dissimilar letters above bars indicate statistical differences between treatments ($P = 0.05$, Duncan's multiple-range test).

explant occurred with 3×10^{-6} M BAP or the BAP + Kin mixture, while 5×10^{-6} M BAP alone inhibited shoot development (Table 1). Removal of the apical bud increased the yield of primary shoots particularly in conjunction with BAP + Kin (Figs. 8 and 9).

Formation of secondary buds

Masses of secondary buds could be induced at the base of each primary shoot after the initial period of incubation (6 weeks) (Fig. 7). Surgical removal of the apical bud significantly increased the production of secondary buds compared with the intact explants (Table 2).

Shoot elongation and multiplication

Shoot elongation was achieved on both half-strength SH medium without cytokinin and on the latter medium containing 1% activated charcoal. The presence of activated charcoal decreased the incubation time from 4–6 to 2–3 weeks. The mean number of secondary shoots produced under shoot-

elongating conditions (without added cytokinin) for 12 weeks was 41, 43, and 34 shoots per cultured explant (without an apical bud) with those that initially received a cytokinin treatment of 10^{-6} M BAP + 10^{-6} M Kin, 3×10^{-6} M BAP, and 5×10^{-6} M BAP, respectively. Duncan's multiple-range test showed that these means were significantly different at the 0.01 level of probability.

When individual primary shoots were maintained on a medium with BAP + Kin for 4–6 weeks, two generations with a multiplication rate of 2–3 times were produced successively within 4–6 weeks.

The same procedure when applied to explants from a 6-month-old seedling induced 98% of the explants to develop axillary buds that developed into vigorous shoots under shoot-elongating conditions.

Discussion

It is generally agreed (e.g., Hussey 1978; Vasil and Vasil 1980; Sharp and Evans 1981) that plants regenerated from shoot tips or buds are genetically stable and free from the somaclonal variation associated with plants differentiated from callus. Evidence from investigations made here in Edinburgh on the performance of plantlets of *Pinus brutia* raised from tissue cultures would support this conclusion. Therefore, it is important to ensure that the primary axillary buds that develop after culture arise directly from existing immature buds without an intervening callus stage. The anatomical evidence presented here show this is indeed the case with *Pinus brutia*. In previous reports (Boulay 1976; Bonga 1977; David *et al.* 1978; Jelaska *et al.* 1981), there would be some doubt as to the exact origin of the buds. It has been shown from this study that the secondary shoots produced arise directly from the primary shoots and not from callus (Fig. 7), although some callus production occurs at the cut end of the explant.

The role of cytokinin is also important. In a previous study, in which organogenesis was stimulated on excised needles of *Pinus brutia*, BAP was found to be more potent than any other cytokinin tested (Abdullah *et al.* 1984). Indeed, in this species, there is also a requirement of BAP for axillary bud formation, although the data presented here reveal that a lower level of cytokinin was required for axillary bud development. This is in contrast to the study of David (1982), who has reported that higher levels of cytokinin (5×10^{-6} M – 10^{-5} M) were required for bud formation in other gymnosperm species. This difference in cytokinin requirement presumably reflects the diversity and physiological state of explants used in other investigations. Indeed, the developmental state of the axillary buds in the material used in the present study could explain why such a low level of cytokinin was sufficient with *Pinus brutia*. It is also

significant that the removal of the apical bud significantly enhanced the yield of primary and secondary buds. Such treatment would remove the dominance of the apical bud over lateral and axillary buds and promote their growth (Kramer and Kozlowski 1979). Since axillary buds under apical dominance have been shown to lack the ability to synthesize their own cytokinin to continue development (Wickson and Thimann 1958), the addition of cytokinin would be expected to stimulate axillary bud development. This study on *Pinus brutia*, that on *Pinus nigra* (Jelaska *et al.* 1981), and the research of David *et al.* (1978) confirm that axillary buds may be activated on pine explants. It has also been shown that the concentration of both potassium and cytokinin in the medium are critical for axillary budding in cotyledons and hypocotyls of *Pinus pinaster* (David *et al.* 1978). In this study, it has been shown that the production of shoots from seedling explants of Calabrian pine was maximized by the simultaneous removal of the apical bud (abolition of apical dominance) and the addition of cytokinin (stimulates axillary bud development). The efficiency of this procedure is also effective with explants taken from older (6-month) plants of *Pinus brutia*; however, in *Pinus pinaster*, a slow and reduced development has previously been recorded in buds produced on explants taken after the cotyledon stage (David *et al.* 1978) and a poor survival rate was reported with isolated shoots developed from axillary buds on hypocotyl explants of *Pinus nigra* (Jelaska *et al.* 1981).

In this study, the production of shoots was not continued beyond the stage described here, but, with both techniques of shoot multiplication, it seems likely that successive generations could be achieved in which the shoot number would increase geometrically. Thus, by applying this procedure it should be possible to produce many thousands of shoots within 1 year.

It seems that the time taken for axillary shoot production in the system described here is much shorter than that with other systems (both primary axillary shoots and secondary buds were developed within 6 weeks). This is clearly an advantage for achieving the goal of commercial production (Sharp and Evans 1981; Thorpe and Biondi 1984).

Rooting of the shoots produced was achieved by treatment with an appropriate combination of growth regulators. Details concerning development, survival, and establishment of regenerated plantlets will be reported in a later publication.

Acknowledgements

The assistance of Dr. Chris Jeffree and John Findlan is gratefully acknowledged.

ABDULLAH, A. A., M. M. YEOMAN, and J. GRACE. 1984. *In vitro* organogenesis of *Pinus brutia* needles and shoot tips. In Proceedings of an International Symposium on Plant Tissue and Cell Culture Application to Crop Improvement, September 24–29, 1984, Prague, Czechoslovakia. Edited by F. J. Novak, L. Havel, and J. Dolezel. Czechoslovak Academy of Sciences, Prague, Czechoslovakia. pp. 177–178.

- . 1985. *In vitro* adventitious shoot formation from embryonic and cotyledonary tissues of *Pinus brutia* Ten. *Plant Cell Tissue Organ Cult.* 5: 35–44.
- ANDERS, O., and M. S. KETTANEH. 1969. Forest tree of Iraq. Mosul University Press, Ninevah, Iraq.
- BONGA, J. M. 1977. Organogenesis in *in vitro* cultures of embryonic shoots of *Abies balsamea* (balsam fir). *In Vitro*, 13: 41–48.
- BORTHWICK, A. W. 1899. On interfoliar buds in pines. *Trans. Proc. Bot. Soc. Edinburgh*, 21: 154–158.
- BOULAY, M. 1976. Recherches sur la propagation du Douglas par culture *in vitro*. *Assoc. For.–Cellul., Ann. Rech. Sylvic.* 1976: 84–145.
- DAVID, A. 1982. *In vitro* propagation of Gymnosperms. In *Tissue culture in forestry*. Edited by J. M. Bonga and D. J. Durzan. Dr. W. Junk Publishers, The Hague, Netherlands. pp. 72–108.
- DAVID, H., K. ISEMUKALI, and A. DAVID. 1978. Obtention de plants de pin maritime (*Pinus pinaster* Sol.) a partir de brachyblastes ou d'apex caulinaires de tres jeunes sujets cultives *in vitro*. *C.R. Seances Acad. Sci., Ser. D*, 287: 245–248.
- FINK, S. 1984. Some cases of delayed or induced development of axillary buds from persisting detached meristems in conifers. *Am. J. Bot.* 71(1): 44–51.
- HUSSEY, G. 1978. The application of tissue culture to the vegetative propagation of plants. *Sci. Prog. (Oxford)*, 65: 185–208.
- JELASKA, S., B. KOLEVSKA-PLETIKAPIC, and M. VIDAKOVIC. 1981. Bud regeneration in *Pinus nigra* embryo and seedling tissue culture. In IUFRO International Workshop on *In Vitro* Cultivation of Forest Tree Species, August 31 – September 4, 1981, Fontainebleau, France. Association Forêt-Cellulose, Paris. pp. 159–165.
- KARNOVSKY, M. J. 1965. A formaldehyde–glutaraldehyde fixation of high osmolality for use in electron microscopy. *J. Cell Biol.* 27: 137A–138A.
- KAYACIK, H., and F. YALTIRIK. 1970. General aspect of Turkish forestry. In *Plant life of South-West Asia*. Edited by P. H. Davis, P. C. Harper, and I. C. Hedge. Aberdeen University Press, Aberdeen, Scotland. pp. 284–291.
- KRAMER, P. J., and T. T. KOZLOWSKI. 1979. Internal factors affecting growth. In *Physiology of woody plants*. Edited by P. J. Kramer and T. T. Kozlowski. Academic Press, New York. pp. 546–627.
- ROLAND, J. C. 1978. General preparation and staining of thin sections. In *Electron microscopy and cytochemistry of plant cells*. Edited by J. L. Hall. North-Holland Publishing Co., Amsterdam, Netherlands. pp. 1–60.
- SHARP, W. R., and D. A. EVANS. 1981. Patterns of plant regeneration: opportunities for cloning or production of genetic variability. *Int. Assoc. Plant Tissue Cult. Newslett.* 35: 2–8.
- STONE, E. L., and M. H. STONE. 1943. Dormant buds in certain species of pinus. *Am. J. Bot.* 30: 346–351.
- THORPE, T. A., and S. BIONDI. 1984. Conifers. In *Handbook of plant cell culture*. Vol. 2. Crop species. Edited by W. E. Sharpe, D. A. Evans, P. V. Ammirato, and Y. Yamada. Macmillan Publishing Co., Riverside, NJ. pp. 435–467.
- VASIL, I. K. and V. VASIL. 1980. Clonal propagation. In *Perspectives in plant cell and tissue culture*. Part A. *Int. Rev. Cytol. Suppl.* 11A: 145–173.
- WICKSON, M., and K. V. THIMANN. 1958. The antagonism of auxin and kinetin in apical dominance. *Physiol. Plant.* 11: 62–74.