

**MICROPROPAGATION OF *ACACIA*
MEARNSII (DE WILLD)**

BY

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PREFACE

The experimental work described in this thesis was conducted in the Botany Department, University of Natal, Pietermaritzburg, from 1996 to 1999 under the supervision of Professor J. van Staden and the co-supervision of Mr. Rob Dunlop from the Institute for Commercial Forestry Research (ICFR).

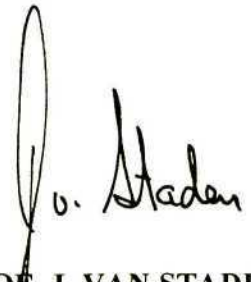
The results have not been submitted in any other form to another University and except where the work of others is acknowledged in the text, are the results of my own investigation.



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NOVEMBER 1999

We certify that the above statement is correct.



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PUBLICATIONS

The following publications were produced during the course of this study :

1. Sascha L. Beck, R. Dunlop and J. van Staden (1998) Micropropagation of *Acacia mearnsii* from *ex vitro* material. Plant Growth Regulation 26:143-148
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ABSTRACT

Multiple shoots were produced from nodal explants of thirty-day-old *in vitro* grown seedlings and from pretreated three, five- and nine-month-old greenhouse grown *Acacia mearnsii* plants, respectively. Explants were sterilized for 15 minutes using 0.1 % HgCl₂ for the three-month-old explants and 0.2 % for the five and nine-month-old explants. Nodal explants were induced to form multiple shoots when placed on Murashige and Skoog (MS) medium supplemented with 2.0 mg l⁻¹ benzyladenine (BA). Rooting of these shoots was achieved on MS medium supplemented with 1.0 mg l⁻¹ indole-3-butyric acid (IBA). Plantlets were acclimatized in transparent plastic containers under greenhouse conditions with a 90 % success rate. These plantlets were successfully acclimatised under greenhouse conditions and planted in the field together with plants regenerated by cuttings.

In an attempt to overcome maturation effects and loss of juvenile characteristics, when using adult plant material *in vitro*, investigations were undertaken into the use of coppice material, as an alternative explant source. *A. mearnsii* trees from five ages (two, four, six, eight and ten-years-old, respectively) were decapitated to a height of 1.5 m. After three weeks, coppice was noted on the stumps of trees from all ages. A linear response to coppice production was noted, with the greatest coppice production being on the two-year-old tree stumps and the least on the ten-year-old tree stumps. Decontamination of the coppice was successful and multiple shoot production was obtained from coppice taken from all age groups on MS medium supplemented with 2.0 mg l⁻¹ BA. The effect of various sucrose concentrations were investigated. Greater shoot production occurred with increased sucrose concentrations (20 and 30 g l⁻¹). It was evident that rejuvenation of mature tissue could be achieved through the use of coppice material.

A second approach to rejuvenate adult material and to overcome the deleterious effects of maturation, was in the use of apical meristems. Meristems were taken from 30-day-old *in vitro* grown plants, from coppice (rejuvenated tissue) and adult material of five various tree ages (two,

four, six, eight and ten-years-old, respectively). Plant material were taken over two seasons (1997 to 1999) and the use of agar and liquid support media were tested under both light and dark conditions. The coppice and adult material was successfully decontaminated in both seasons. In the first season (1997/1998), shoot production was obtained from meristems of *in vitro* grown plants, coppice and adult material from all trees on MS medium alone or MS medium supplemented with 2.0 mg l^{-1} BA. In the following season (1998/1999), the use of a solidified agar medium was superior to the use of a liquid culture. There appeared to be no significant difference ($p < 0.05$) between the use of light or dark culture conditions. Various media were tested and maximum shooting occurred on half-strength MS medium and Woody Plant Medium (WPM). However, once multiple shoot primordia were initiated, shoot elongation posed a problem. It was for this reason that the size of the meristems excised from the coppice material was increased from 0.5 mm to 1.0 mm in the 1997/1998 season, to 1.0 to 2.0 mm in the 1998/1999 season. The use of gibberellic acid and 100 ml jars were also investigated to see if this might enhance shoot elongation. Sufficient plant material was not available for a thorough investigation. Environmental conditions under which the plant material (adult or coppice) was harvested was similar in both seasons, with respect to temperature, but differed in rainfall. Rainfall was high (105.1 mm) in 1997/1998 season and low (ranging from 59.8 to 71.45 mm) in the 1998/1999 season. Shoot production from meristems taken from coppice material in the 1998/1999 season was significantly greater ($p > 0.05$) than that in the 1997/1998 season, whereas shooting from the adult plant material remained unchanged. The disadvantage with using coppice material is that its production on decapitated tree stumps is dependant on rainfall, which is unpredictable. The differences in results from coppice material could be attributed to the fact that the trees felled in the two seasons were not related to each other in any way. In both seasons meristems, tree age was not a limiting factor, for meristems from adult and from coppice material. Meristems from the ten-year-old trees were as productive as those taken from the two-year-old trees. In the 1997/1998 season the results from the meristems from the adult material was equal if not greater than those obtained from the coppice material. In the 1998/1999 season, there was no significant difference ($p < 0.05$) in percentage shoot production between the meristems from the adult and coppice material throughout the age groups. This suggests that the use of rejuvenated tissue in the form of coppice is not essential. This re-emphasized the advantage of using meristems taken from adult plant material.

This study provided suitable protocols for the micropropagation of both *in vitro* and *ex vitro* grown nodal explants of *A. mearnsii*. However, as the plant material obtained from the field matures so the ease of obtaining sterile material decreased, thus reducing the chances of *in vitro* micropropagation. For this reason suitable pretreatments and rejuvenation methods are necessary if explants from mature field tissue are to be introduced into culture and successfully micropropagated. This study has shown that through the use of nodal material (taken from coppice produced on adult tree stumps) and apical meristems taken from both coppice and mature plant material, adult material can be successfully decontaminated, introduced into culture and stimulated to produce shoots.

Analysis of tannin production was conducted to see if there was any indication that the presence of tannins in the plant material effected *in vitro* culture of nodal explants. However, no trends were obtained suggesting any influence of tannins on *in vitro* performance.

In future years after further optimisation, these techniques could be incorporated in an *A. mearnsii* clonal programme, with the advantage of possibly eliminating maturation effects, commonly noted in vegetative practices. This will allow for easy manipulation and amplification of superior quality adult material.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of variance
B ₅	GAMBORG B ₅ medium (in GAMBORG, MILLER and OJIMA, 1968)
BA	Benzyladenine
BD	BONNER and DEVERIAN (1939) medium
CM	Coconut milk
CW	Coconut water
2,4-D	2,4-Dichlorophenoxyacetic acid
240	Modified PULLMAN and WEBB (1994) medium
GA ₃	Gibberellin A ₃ ; gibberellic acid
IAA	Indoleacetic acid
IBA	Indolebutyric acid
ICFR	Institute for Commercial Forestry Research
KT	KATHJU and TEWARI (1973) medium
LS	LIN and STABA (1961) medium
LSD	Level of significant difference
MS	MURASHIGE and SKOOG (1962) medium
NAA	α -Naphthaleneacetic acid
SH	SCHENK and HILDERBRANDT (1972) medium
WPM	Woody Plant Medium (LLOYD and McCOWN, 1981)
Q-LP	QUOIRIN and LEPOIVRE (1977) medium

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CHAPTER 1

INTRODUCTION

1.1 The importance of the black wattle (*Acacia mearnsii*)

The genus *Acacia* comprises many species which are important for firewood, fodder, shelterbelts and soil improvement. Species of *Acacia* are dispersed widely in tropical and subtropical regions of Australia, South America, Asia and Africa (JONES, BATCHELOR and HARRIS, 1990). The black wattle (*Acacia mearnsii*) was introduced into South Africa in 1864 by John Vanderplank as seed obtained from Australia (STUBBINGS and SCHÖNAU, 1994). The success of early plantings of black wattle, particularly in South Africa, stimulated interest in the tree as a plantation crop in other parts of Africa and elsewhere in the world. It has been grown primarily for the tannin in its bark, and also as an additional source of fuel and timber, in some cases to reduce the drain on natural forests in certain countries. Its rapid growth and potential soil-improving qualities as a legume have led to its use in combatting soil erosion.

Wattle, also known as Mimosa, is one of a number of sources of tannins for the vegetable tanning industry in the world. Historically, the main product from black wattle was the bark, which is used directly or in a ground form as a tanning extract (HEIBERG-IURGENSEN, 1967). The bark is one of the richest vegetable tanning materials. When dried, 35 % of its weight consists of tannin (BEARD, 1957). By 1960, approximately 300 000 hectares were planted to black wattle in South Africa, however, the bark market has fallen and the area is now in the region of 130 000 hectares. In the 1970's the use of black wattle timber as pulpwood and for charcoal production became an important additional element in the economics of wattle growing (STUBBINGS and SCHÖNAU, 1994). Black wattle timber, apart from its value as fuel, in tannin production and as mine timber, is also suitable for the production of parquet blocks, charcoal, hardboard, rayon and certain types of paper (SHERRY, 1971). In recent years black wattle timber has become extremely popular as a source of high quality pulp. In the last decade wattle timber has become extremely valuable and its importance as a source of pulpwood both locally and internationally, has risen. South

Africa per annum, produces approximately 42856 tons of wattle mining timber ; 416098 tons of paper ; 123104 tons of pulpwood ; 242561 tons of wood chips ; 27778 tons of charcoal ; 81692 tons of firewood and 21926 tons of other by-products (Department of Water Affairs and Forestry, 1997/1998). The production of improved seed and the maintenance of a high level of productivity is particularly important to the wattle industry of South Africa. It is for this reason that the black wattle has become an important topic of vegetative propagation research.

1.2 Methods of propagation

The majority of forest trees have in the past been propagated through the traditional family forestry method, where trees are grown from seed and propagated sexually. Superior stocks have been propagated vegetatively through the use of rooted cuttings and grafting. The recent large scale cloning of spruces and eucalypts has validated the importance of clonal propagation. Cloning allows for the immediate and total capture of genetic gain. The effect of age of the plant material restricts this technique. For example, the ability of a cutting to produce roots, decreases with age. The growth effects of such propagules differ with age, species and genotype of the plant. It also varies with position in the crown of the plant material from which the cutting was taken and the time of year (FRANCLET, BOULAY, BEKKAOUI, FOURET, VERSHOORE-MARTOUZET and WALKER, 1987).

Acacia mearnsii reaches maturity in approximately ten years (BEARD, 1957), thus progress in attempting to achieve improved seed for wattle growers, has been slow due to its long rotation age and limited due to the poor rooting abilities of the wattle. Clonal propagation through tissue culture offers an alternative to vegetative practices used in the past and has the potential to provide high multiplication rates of uniform genotypes, resulting in short term gains (GUPTA, PULLMAN, TIMMIS, KREITINGER, CARLSON, GROB and WETTY, 1993). Clonal propagation of mature tissues through tissue culture allows for the improved quality of selected traits such as high yield and superior pulping properties (JONES and VAN STADEN, 1997). Tissue culture also allows for large amounts of material to be produced, therefore allowing for intensive selection (TIMMIS, ABO EL-NIL and STONECYPHER, 1987). Tissue culture techniques have been applied to wide range of tree species. It is evident

that there is a strong and intricate interaction between the explant, plant growth regulators, culture conditions and genotype (NEHRA and KARTHA, 1994). However, for these procedures to be commercially viable the post-culture performance in the field must be improved as the initial financial outlay is high (JONES and VAN STADEN, 1997).

The wattle industry in South Africa is an important source of employment and export capital (SHERRY, 1971). For many years South African wattle has been amongst the best in the world, yielding high quality bark extract and allowing the South African Wattle Industry to be a major player in the world market of vegetable tannin extract. Other wattle products have also become important commodities. Recently, countries such as China and Brazil have invested large sums of money into research pertaining to black wattle and other species that have high tannin yields. To maintain the competitiveness of the South African Wattle Industry it is necessary to develop more rapid methods of propagating the most superior genotypes available. For this reason clonal propagation through tissue culture could be the solution. Tissue culture is however, initially very costly. Only once the technique has been developed for the specific plant material and the method is efficient, can it be cost effective.

1.3 Aims and Objectives

It is well known that there is a need to produce improved wattle seed to be grown commercially in South Africa. Genetic improvement of black wattle has taken place at the Institute for Commercial Forestry Research (ICFR (earlier WRI)) for the past 50 years, with gains being made with respect to disease resistance, tree volume, bark quality and quantity and stem form. Due to the poor rootability of the species and low success rates when grafted, the clonal option in the improvement programme has been limited. It is therefore evident that emphasis needs to be placed on initiating a suitable clonal programme for the wattle. Due to the economic importance of this forest tree and due to limited *in vitro* research done on this species, it was decided to investigate techniques involving vegetative reproduction through micropropagation, with the aim of ultimately introducing mature field grown plant material into culture. This would allow for rapid multiplication of superior clones and will eliminate the affects of maturation, thus enabling desirable adult material to be mass propagated with ease.

The objectives of the studies were to increase the vegetative reproduction rate and rootability of *Acacia mearnsii* by investigating tissue culture techniques such as organogenesis. Due to the limited research done on the black wattle in particular, experiments were based on previous work done on other *Acacia* species. Plantlets were grown from seed *in vitro* and used as a source of explants. Once a suitable explant, culture medium and hormone concentration for optimal *in vitro* plantlet regeneration was identified, the findings were applied to adult tissue. The main emphasis of the research was subsequently aimed at introducing adult tissue into culture as cloning of mature trees is generally preferred over juvenile tissues. There are, however, a number of problems associated with adult tissue which restricts *in vitro* propagation. Mature tissue has characteristics of poor rooting, reduced vigour and elevated levels of internal contamination thus reducing its chances of success in culture. For this reason, the later research was aimed at rejuvenating mature tissue through the use of coppice material and through meristem culture.

The use of rejuvenated adult tissue as a source of explant for *in vitro* culture could prove to be a novel means of introducing mature wattle tissue into culture. Which could in turn be used in future clonal programmes. Trees from five age groups were selected, namely, two, four, six, eight and ten years of age. Experiments were conducted over two growing seasons. Meristem culture and *in vitro* regeneration from nodal explants was conducted on material from both adult trees and coppice material from each of the five age groups. Thus allowing for a comparison between adult and juvenile material for each age group and between age groups.

The ICFR kindly provided plant material and have marked selected trees for felling, which were used for coppicing experiments. Once *in vitro* plantlet regeneration was achieved, these plantlets were acclimatised and introduced into field trials, in order for comparison with seed grown plants and plants propagated through cuttings. Bark samples were collected from each of the five age groups and tannin analysis was conducted. The effect of tannins on *in vitro* regeneration abilities was investigated along with testing to see if tannin production could be an indicator of the transition from the juvenile to mature phase.

With respect to *Acacia* species in particular, the use of tissue culture practices in a breeding programme, have only been attempted with *A. melanoxylon*, where *in vitro* culture was used to provide early amplification of limited seed (JONES, SMITH, GIFFORD and NICHOLAS, 1991). Little work and success have been achieved with *A. mearnsii*. GONG, AL-KHAYRI and HUANG (1991), showed callus production on hypocotyl explants. HUANG, AL-KHAYRI and GBUR (1994) micropropagated *A. mearnsii* from shoot tips originating from three-week-old *in vitro* grown seedlings. No further research has been documented. Due to the economic importance of the black wattle and the need to establish a successful clonal programme for it, the results obtained from this study are particularly important.

The methods and approaches to this study are stated and discussed in the following Chapters.

CHAPTER 2

LITERATURE REVIEW

2.1 The need for clonal forestry

The biodiversity of world forests is declining at a rapid rate, however, the demand for wood is proposed to double in the next century (GUPTA, PULLMAN, TIMMIS, KREITINGER, CARLSON, GROB and WETTY, 1993). South Africa has relatively sparse natural forests and timber resources, however, it has developed into a world leader in plantation forestry (MACRAE, 1994). The forest industry is vital to the economies of many countries.

Reforestation requires tree improvement and emphasis has shifted to the production of genetically superior trees with the aim of producing faster growing trees with improved dimensions, increment, better wood quality, disease resistance and environmental tolerance (DURZAN, 1988).

Forests need to be replenished as they are continually being harvested. Global problems, increasing populations and diminishing resources, necessitates improvement in the strategies of breeding of woody perennials. There is a need to protect current resources against genetic erosion, insects, disease, fire and other natural disasters. Vegetative propagation is a pivotal technology, where large trees with long, complex life cycles are brought into the laboratory for improving productivity and for clonal propagation. Genetic gains can be maintained by clonal propagation and new variation can be introduced through new biotechnological techniques (DURZAN, 1988).

Clonal propagation by tissue culture offers an alternative to cuttings and has the potential to provide high multiplication rates of uniform genotypes resulting in short term gains (DURZAN, 1988). Currently most of the improved germplasm comes from long-term breeding programmes, which are normally costly. Some important trees can now be selected, rooted, micropropagated, grafted, rejuvenated and cloned by somatic embryogenesis and polyembryogenesis (DURZAN, 1988). Clonal forestry is receiving increased recognition as an alternative to conventional vegetative practices (HAN, SHIN and KEATHLEY, 1997).

Cutting propagation is not always successful with older material due to poor rooting (a maturation effect). Micropropagation has provided an alternative for species which are difficult to root. This method may be expensive, however, it can provide stock plants for further multiplication (JONES, 1986). Planting genetically superior clones instead of seedlings, which vary both genetically and phenotypically, may increase forest productivity. The advantage of micropropagated trees is that they have juvenile characteristics such as rapid growth. The trees are usually more uniform in height, and trunk girth, and show reduced bark fissuring (a mature characteristic). Some tissue cultured trees flower earlier than seedling-derived trees (HAN, SHIN and KEATHLEY, 1997). Tissue culture also allows for trees to be genetically engineered. At the same time it allows for the acceleration of improvement programmes and for the rapid exploitation of genetic gain (HAN, SHIN and KEATHLEY, 1997).

Presently importation of cuttings and rooted stock involves lengthy delays due to quarantine procedures to control the spread of disease. Tissue culture may provide a more efficient means of importing selected clones. Disinfected, *in vitro* cultured material would reduce the chances of introducing foreign pathogens (JONES, 1986) and allow for the importation of desirable clones *in vitro* to various countries, for selection work (JONES, SMITH, GIFFORD and NICHOLAS, 1991).

2.2 Clonal propagation of *Acacia* species

In the past considerable research has been conducted on *Acacia* species and varying degrees of plantlet regeneration has been achieved using various explants of a number of *Acacia* species. For example explants such as shoot tips of *A. koa*, cotyledonary buds of *A. albida*, shoot cultures of mature plants of *A. ligulata*, stem cultures of *A. senegal*, nodes and internodes of field grown twigs of *A. nicolata*, shoot tips, axillary buds and hypocotyls of *A. auriculiformis*, shoots from juvenile *A. melanoxylon*, hypocotyl explants from seedlings of *A. mearnsii* and shoots from *in vitro* propagated *A. mearnsii*, have resulted in various degrees of plantlet regeneration (Table 2.1) (GASPAR and COUMANS, 1987). The problem with the propagation of hardwoods, is their recalcitrance to form adventitious

roots. DARUS (1991a) noticed that for root formation, excised micropropagated shoots of *A. mangium*, which were treated with a hormone rooting powder (Seradix no.3), produced more roots than when the shoots were treated with the auxins NAA and IAA (DARUS,1991a). DAS, CHAKRAVARTI and MAITY (1993) took shoots of *A. auriculiformis* and rooted them using MS medium supplemented with 1.0 or 1.5 mg l⁻¹ IBA. TODA, TAJIMA and BRINI (1995) rooted shoots from *A. mangium* on MS medium supplemented with IBA and/or NAA. HUANG, AL-KHAYRI and GBUR (1994) rooted shoots generated *in vitro* on half-strength MS medium supplemented with 0.6 mg l⁻¹ NAA.

Clonal forestry is now recognized as an alternative to conventional vegetative practices (HAN, SHIN and KEATHLEY,1997). *In vitro* cloning of mature trees is generally preferred over juvenile tissues as it is not always possible to determine if the juvenile tissues will have the desired qualities when they mature (BONGA,1987). Establishment of cultures *in vitro* using mature tissue is often problematic (JONES and VAN STADEN,1997). Successful *in vitro* establishment of explants from adult *Acacia* trees has been achieved for *A. auriculiformis* (WANTANABE, IDE and IKEDA,1994 ; REDDY, VEERANAGOUDA, PRASAD, PADMA, UDAYAKUMAR and PATIL,1995 ; TODA, TAJIMA and BRINI,1995 ; ZHANG, HUANG, FU, YANG and CHEN,1995), *A. mangium* (TODA, TAJIMA and BRINI,1995 ; ZHANG, HUANG, FU, YANG and CHEN,1995) and *Robinia pseudoacacia* (HAN, SHIN and KEATHLEY,1997). In the above species, axillary buds were used as explants and the results varied from 28 % shoot production for axillary buds from two-year-old *A. auriculiformis* seedlings (TODA, TAJIMA and BRINI,1995), to 71 % for buds from two-year-old *A. mangium* seedlings (TODA, TAJIMA and BRINI,1995). The problem with the propagation of hardwoods is their difficulty in rooting (an effect of maturation). Once the trees are ready for genetic evaluation they are often recalcitrant (JONES and VAN STADEN,1997). Since it is easier to propagate juvenile tissues (THORPE, HARRY and KUMAR,1991), rejuvenation and the establishment of juvenile characteristics prior to *in vitro* culture, has proved to be important (JONES and VAN STADEN,1997).

Table 2.1 Summary of information relating to the *in vitro* culture of *Acacia* species. Where no values are indicated, the authors did not provide the required experimental detail.

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>Acacia albida</i>	cotyledon with stem piece	multiplication of cotyledon buds	MS	15	(0.5) NAA + (3) BA	DUHOUX and DAVIES (1985)
<i>A. albida</i>	axillary buds of shoots	100 % rooted	LS	50		DUHOUX and DAVIES (1985)
<i>A. albida</i>	nodes from suckers	rooted plants	MS		(0.5) BA + (0.01) NAA + (20000) activated carbon	GASSAMA (1989)
<i>A. albida</i>	rooted plantlets	axillary shoots	MS		(20) BA	GASSAMA (1989)
<i>A. albida</i>	excised roots	root growth	BD	20	(9) Myo-inositol	AHEE and DUHOUX (1994)

<i>Acacia species</i>	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. albida</i>	root	shoots	1/5 MS			AHEE and DUHOUX (1994)
<i>A. albida</i>	shoots	shoots grew and rooted	MS		(0.02) NAA	RUREDZO and HANSON (1993)
<i>A. auriculiformis</i>	axillary buds of seedlings	shoots	B ₅		(0.2) BA + (50000-1000000) CM	MITTAL et al. (1989)
<i>A. auriculiformis</i>	shoots	roots at base	B ₅		(0.02) IAA or (0.01-0.02) NAA	MITTAL et al. (1989)
<i>A. auriculiformis</i>	hypocotyl	shoots	MS	30	(1) BA and (0.5) NAA + (1) BA and glutamine	RANGA RAO and PRASAD (1991)
<i>A. auriculiformis</i>	shoots	roots and plantlet regeneration	half MS	30	(1) IBA and (1) IBA + (0.5) NAA	RANGA RAO and PRASAD (1991)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. auriculiformis</i>	bud from branch-cutting of seedling; sprouting buds from stem cuttings	multiple shoot induction	MS		(2.3) BA + (0.1) IBA	SEMSUNTUD and NITIWATTANACHAI (1991)
<i>A. auriculiformis</i>	shoot	root induction	WHITE (1963)		(0.4) IBA + (0.2) IAA or (0.2) IBA + (0.4) NAA	SEMSUNTUD and NITIWATTANACHAI (1991)
<i>A. auriculiformis</i>	shoot and axillary bud	shoot growth and elongation	half MS		(0.02) NAA + (1) BA + GA ₃	WANTANABE et al. (1994)
<i>A. auriculiformis</i>	shoot	rooting	half MS		hormone free or (0.02) NAA	WANTANABE et al. (1994)
<i>A. auriculiformis</i>	epicotyl	elongation of main shoot	half MS		(5) GA ₃ + (0.02) NAA	IDE et al. (1994)
<i>A. auriculiformis</i>	shoot	growth of axillary shoot	half MS		(1.5) BA	IDE et al. (1994)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. auriculiformis</i>	cotyledon axillae	shoots	half MS		(2) BA	IDE et al. (1994)
<i>A. auriculiformis</i>	shoot	roots	half MS		hormone free or with (0.02) NAA	IDE et al. (1994)
<i>A. auriculiformis</i>	cotyledon callus	shoot primordia	MS	20	BA (concentrations not stated)	DAS et al. (1993)
<i>A. auriculiformis</i>	shoot	leaflets and leaf phyllodes	MS	20	BA (concentrations not stated)	DAS et al. (1993)
<i>A. auriculiformis</i>	shoot	rooting	MS	15	(1) or (1.5) IBA	DAS et al. (1993)
<i>A. auriculiformis</i>	axillary buds of 20-year-old trees	Nodal shoots and plantlet regeneration.	MS		Combinations of GA ₃ , NAA and IBA (concentrations not stated)	REDDY et al. (1995)
<i>A. auriculiformis</i>	hypocotyls from germinating seedlings	80 to 100 % shoot production	half MS		(1 - 2) BA	TODA et al. (1995)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. auriculiformis</i>	shoot	17.7 % rooting	half MS		IBA and NAA (concentrations not stated)	TODA et al. (1995)
<i>A. auriculiformis</i>	axillary buds of two-year-old seedlings	57.1 % shoot production	MS		(1) BA	TODA et al. (1995)
<i>A. auriculiformis</i>	axillary buds of two-year-old seedlings	28.6 % shoot production	half MS		(1) BA	TODA et al. (1995)
<i>A. auriculiformis</i>	shoots	38.5 % rooting	MS		(2000) NAA	TODA et al. (1995)
<i>A. auriculiformis</i>	small pieces of shoot and axillary buds of one-year-old seedlings	shoot proliferation and elongation	half MS		(0.02) NAA + (1) BA Addition of GA ₃ helped slightly to shoot elongation	WANTANABE et al. (1994)
<i>A. auriculiformis</i>	shoot	rooting	half MS		(i) Hormone free (ii) 0.02 NAA	WANTANABE et al. (1994)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. auriculiformis</i>	buds from four-year-old trees (large explants)	66.7 % gave multiple shoots. 55 shoots produced	MS			ZHANG et al. (1995)
<i>A. auriculiformis</i>	shoot	29.8 % rooting	MS			ZHANG et al. (1995)
<i>A. auriculiformis</i>	axillary buds of <i>in vitro</i> grown seedlings	multiple shoots	B ₅		CW + BA (concentrations not stated)	GUPTA and AGRAWAL (1992)
<i>A. auriculiformis</i>	shoot	rooting	B ₅		CW + NAA / IAA (concentrations not stated)	GUPTA and AGRAWAL (1992)
<i>A. auriculiformis</i>	rooted shoots	acclimatized, flowered and set seed	in the field			GUPTA and AGRAWAL (1992)
<i>A. bivenosa</i> <i>A. holosericea</i>	node, internode and phyllode	callus and axillary shoot growth from nodal explants	MS		IAA or IBA + BA (concentrations not stated)	JONES et al. (1990)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. bivenosa</i> <i>A. holosericea</i>	axillary shoots	roots	MS		(1.8) IBA	JONES et al. (1990)
<i>A. catechu</i>	immature cotyledons	callus	WPM		(3) kinetin + (0.5) NAA	ROUT et al. (1995)
<i>A. catechu</i>	callus	somatic embryos and secondary embryogenesis	WPM		(3) kinetin + (0.5) NAA + (104-4029) 1-proline	ROUT et al. (1995)
<i>A. catechu</i>	somatic embryos	germination	half MS	20		ROUT et al. (1995)
<i>A. koa</i>	seedling shoot tip	callus	SH	30	(0.2) 2,4-D	SKOLMEN and MAPES (1976)
<i>A. koa</i>	explant and callus	callus growth	MS	30	(2.5) 2,4-D + (10000) CM	SKOLMEN and MAPES (1976)
<i>A. koa</i>	callus	shoot primordia in callus	MS	30	(10000) CM	SKOLMEN and MAPES (1976)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. koa</i>	callus and primordia	leaf primordia	MS	30	(5) BA	SKOLMEN and MAPES (1976)
<i>A. koa</i>	shoot	pre-elongation culture	MS	30	(10000) CM	SKOLMEN and MAPES (1976)
<i>A. koa</i>	shoot	shoot elongation	MS	30	(1) BA	SKOLMEN and MAPES (1976)
<i>A. koa</i>	shoot	root system initiated	MS	30	(0.2) IBA	SKOLMEN and MAPES (1976)
<i>A. koa</i>	shoot	rooted plant and shoots	MS	30		SKOLMEN and MAPES (1976)
<i>A. ligulata</i>	adventitious or axillary shoots	77 % shoots rooted	MS	10		WILLIAMS et al. (1985)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. mangium</i>	nodes from germinated seedlings	shoot growth	MS	30	(0.5) BA	DARUS (1991a)
<i>A. mangium</i>	shoots	root formation	MS	30	Seradix 3 (concentrations not stated)	DARUS (1991a)
<i>A. mangium</i>	nodes from two-week-old and seven-month-old explants	shooting	MS	30	(1-2) BA	GALIANA et al. (1991)
<i>A. mangium</i>	shoot	rooting	half MS	30	(0.05) IBA	GALIANA et al. (1991)
<i>A. mangium</i>	hypocotyls from germinating seedlings	80 to 100 % shoot production	half MS		(1 - 2) BA	TODA et al. (1995)
<i>A. mangium</i>	shoot	62.5 % rooting	half MS		IBA and NAA (concentrations not stated)	TODA et al. (1995)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. mangium</i>	axillary buds of two-year-old seedlings	71.4 % shoot production	MS		(1) BA	TODA et al. (1995)
<i>A. mangium</i>	axillary buds of two-year-old seedlings	85.7 % shoot production	half MS		(1) BA	TODA et al. (1995)
<i>A. mangium</i>	shoot	69.2 % rooting	MS		(2000) NAA	TODA et al. (1995)
<i>A. mangium</i>	buds from four-year-old trees	60 % produced multiple shoots. 48 shoots produced.	MS			ZHANG et al. (1995)
<i>A. mearnsii</i>	hypocotyl	callus	MS	30	(1000) Casein hydrolysate + (3) NAA + (1) BA	GONG et al. (1991)
<i>A. mearnsii</i>	<i>in vitro</i> grown shoot tip	shooting	MS		(2) BA	HUANG et al. (1994)
<i>A. mearnsii</i>	shoot	rooting	half MS		(0.6) NAA	HUANG et al. (1994)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. melanoxylon</i>	shoots (axillary bud and piece of shoot)	callus + shoots	MS	20	(0.2) BA + (0.2) IAA	MEYER and VAN STADEN (1987)
<i>A. melanoxylon</i>	shoot	rooted shoots	MS	20	(2) IBA	MEYER and VAN STADEN (1987)
<i>A. melanoxylon</i>	nodes	axillary bud development	MS		(1) BA alone or with (0.5) NAA	JONES and SMITH (1988)
<i>A. melanoxylon</i>	embryos	shoot and root development	Q-LP			JONES (1986)
<i>A. melanoxylon</i>	Shoot	rooting	Q-LP		(5) IBA + (2.5) NAA + (0.2) BA	JONES (1986)
<i>A. nilotica</i>	cotyledon nodes	shoots	B ₅		(1.5) BA	DEWAN et al. (1992)
<i>A. nilotica</i>	shoot	roots	B ₅		(2) IAA	DEWAN et al. (1992)

<i>Acacia</i> species	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. nilotica</i>	stem	shoot buds and roots	MS		(0.5 - 1) IAA	MATHUR and CHANDRA (1983)
<i>A. nilotica</i>	axillary buds from <i>in vitro</i> grown seedlings	multiple shoots	B ₅		CW + BA (concentrations not stated)	GUPTA and AGRAWAL (1992).
<i>A. nilotica</i>	shoot	roots	B ₅		CW + NAA / IAA (concentrations not stated)	GUPTA and AGRAWAL (1992).
<i>A. nilotica</i>	rooted shoots	acclimatized, flowered and set seed.	in the field			GUPTA and AGRAWAL (1992).
<i>A. salicina</i> <i>A. saligna</i> <i>A. sclerosperma</i>	node, internode, phyllode	callus and axillary shoot growth	MS		IAA or IBA + BA	JONES et al. (1990)
<i>A. salicina</i> <i>A. saligna</i>	axillary shoot	roots	MS		(1.8) IBA	JONES et al. (1990)

<i>Acacia species</i>	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. saligna</i>	shoot tips	multiple buds	MS		(5-9) BA	BARAKAT and EL-LAKANY (1992)
<i>A. saligna</i>	buds	shoot elongation	MS		(0.3) BA + (0.2) IAA	BARAKAT and EL-LAKANY (1992)
<i>A. saligna</i>	shoot	rooted	MS		(2) IBA	BARAKAT and EL-LAKANY (1992)
<i>A. senegal</i>	seedling cotyledon	rhizogenesis	KT	40	(2) NAA + (2) 2,4-D	KATHJU and TEWARI (1973)
<i>A. senegal</i>	explants	shoot development	half MS		(11) zeatin	BADJI et al. (1993)
<i>A. senegal</i>	shoots	root initiation	half JORDAN'S		(9) NAA	BADJI et al. (1993)
<i>A. senegal</i>	roots	root extension	half JORDAN'S			BADJI et al. (1993)

<i>Acacia species</i>	Explants used	Results obtained	Medium used	Sucrose (g l ⁻¹) added	PGR's (mg l ⁻¹) and other supplements	Reference
<i>A. tortilis</i>	cotyledon nodes	shoot bud formation	MS		(0.1) NAA + (5) BA	NANDWANI (1995)
<i>A. tortilis</i>	shoot	rooting	half MS		(3) IBA	NANDWANI (1995)
<i>Robinia pseudoacacia</i>	buds from 15 and 25-year-old trees	shooting	MS		(0.2) BA	HAN et al. (1997)
<i>Robinia pseudoacacia</i>	shoot	rooting	half MS		(0.2) IBA	HAN et al. (1997)
<i>Rose acacia</i>	cambium	callus	MS		(1) 2,4-D	DEGUCHI et al. (1994)
<i>Rose acacia</i>	callus	adventitious buds	liquid half MS		(1) IAA or (0.1) BA	DEGUCHI et al. (1994)
<i>Rose acacia</i>	buds	shoots	half MS		(0.1) BA	DEGUCHI et al. (1994)
<i>Rose acacia</i>	callus	rooting	liquid half MS		(1) IAA	DEGUCHI et al. (1994)

2.3 Juvenility versus maturity

Establishment of cultures from adult tissue is problematic and thus rejuvenation has become an important process for *in vitro* culture (JONES and VAN STADEN, 1997). *In vitro* propagation could allow for rapid introduction of new clones bearing desirable characteristics or be used for mass production of uniform and pathogen-free plants. However, regeneration *in vitro* of proven genotypes, which are usually from mature individuals, has a low success rate despite its importance for clonal propagation (ECONOMOU and SPANOUDAKI, 1988). Problems with low reactivity of mature tissue, apical necrosis and phenolic compounds, need to be overcome in order to succeed with the *in vitro* multiplication of adult material (CHAUVIN and SALESSES, 1988). Cloning of mature trees is generally preferred over juvenile tissues as it is not always possible to determine if the juvenile tissues will have the desired qualities when they mature (BONGA, 1987). By the time the trees are mature enough for evaluation, they are often recalcitrant to rooting (JONES and VAN STADEN, 1997). For this reason tissue culture techniques in many species have been restricted to the use of juvenile material. This limits the technique as an alternative to traditional vegetative propagation. This problem can be avoided by using explants which retain juvenile characteristics such as epicormic shoots or shoots from the lower part of the trunk (HAN, SHIN and KEATHLEY, 1997). Since it is easier to propagate juvenile tissues (THORPE, HARRY and KUMAR, 1991) rejuvenation and the establishment of juvenile characteristics prior to *in vitro* culture, has proved to be important. Rejuvenation can be achieved through pruning, grafting and the stimulation of axillary shoots (JONES and VAN STADEN, 1997). When rejuvenating mature tissues the most juvenile tissue and the smallest explant possible should be used so as to disrupt the controlling forces of the mother plant. These controlling forces are reduced by pretreatment of the plant and through the use of repeated culture. Tissues which have a known fixed pattern of development should be avoided and the culture conditions should suit the explant being used (BONGA, 1987). Previous studies have shown that the age of the plant material plays a determining role, and that the maturation process has a negative effect on rooting potential (POUPARD, CHAUVIE RE and MONTEUUIS, 1994).

In trees, both juvenile and mature phases may occur on the same adult tree. The upper parts of a tree such as the apical tip are younger, however, they show maturation affects, whereas plant growth on the lower and older parts, frequently retain juvenile traits (HAN, SHIN and KEATHLEY, 1997). Terminal shoot cuttings are more responsive to rooting than nodal cuttings. However, adventitious rooting abilities of such material varies according to the age of the mother plant (MONTEUUIS, VALLAURI, POUPARD and CHAUVIE`RE, 1995). The potential for adventitious bud and root initiation (a juvenile characteristic) seems to be inversely related to the phase change from juvenility to maturity (HACKETT, 1987). SANCHEZ and VIETEZ (1991) noted that cultures of chestnut explants taken from the crown branches, produced longer but fewer shoots than explants taken from basal material (MONTEUUIS, VALLAURI, POUPARD and CHAUVIE`RE, 1995). ECONOMOU and SPANOUDAKI (1988) took leaf-less shoots from a 35-year-old oleaster tree (a woody plant) and noted that basal parts of the explants produced more axillary shoots than the upper-parts (4.0 and 1.6 being the average number of shoots produced, respectively). This could possibly be due to a release from apical dominance (ECONOMOU and SPANOUDAKI, 1988). In eucalypt seedlings the cotyledonary node has a high rooting potential. This, however, decreases as the distance from the cotyledonary node increases. It has also been shown that cuttings taken from shoots formed at the basal region have a greater rooting potential than those taken from shoots formed at the upper part of the plant. Thus juvenile characteristics, such as bud formation and rooting potential may be preserved at the base of the plant in young meristematic tissue, while maturation occurs in the periphery of the plant in phase transitionally older, but chronologically younger tissues (HACKETT, 1987). MONTEUUIS, VALLAURI, POUPARD and CHAUVIE`RE (1995) showed that cuttings taken from the crown of a four-year-old *A. mangium* tree had reduced rootability compared with sprouts taken from four-year-old stumps. POUPARD, CHAUVIE`RE and MONTEUUIS (1994) showed that sprouting shoots from a mature stump of *A. mangium*, close to the root system, usually considered the most juvenile and responsive, actually had lower rooting rates than six-month-old seedlings which in turn had a weaker overall rooting rate than 56-day-old seedlings. According to HACKETT (1988) the difference in rooting between stump sprouts and six-month-old seedlings might be due to a deficiency in endogenous promoters or to an excess of inhibitors in the mature plant material (POUPARD,

CHAUVIE`RE and MONTEUUIS,1994). Several attempts have been made to understand the physiological gradient in maturity *in vitro*. SANCHEZ and VIETEZ (1991) compared *in vitro* reactivity between explants from the crown branches and from basal sprouts. It was found that the latter were more responsive *in vitro* (HAN, SHIN and KEATHLEY,1997).

Rejuvenation occurs in two specific areas in a tree, namely the meristematic structures and at the base of the trunk (FRANCLET, BOULAY, BEKKAOUI, FOURET, VERSHOORE-MARTOUZET and WALKER, 1987). Rejuvenation of shoot cuttings has been established by grafting mature shoots onto juvenile root stocks and through pruning. Such rejuvenation methods have shown that improved rooting occurs through the use of serial cutting processes (JONES and VAN STADEN,1997). Cuttings from the base of the trunk, orthotropic shoots from the base of the trunk and shoots from sphaeroblasts are more juvenile than other parts of the plant. Thus, the use of stump shoots and the practice of coppicing are commonly used methods (THORPE, HARRY and KUMAR,1991). Delayed maturation is best noted in stump sprouts. These generally arise from buds which develop in the bark of the stem of seedlings and they remain dormant. These shoots are juvenile and thus easy to root. Stump sprout explants exhibit better stem elongation and a greater rooting ability than shoots regenerated from the crown (BONGA and VON ADERKAS, 1992). MONTEUUIS, VALLAURI, POUPARD and CHAUVIE`RE (1995) showed that mature *A. mangium* can be successfully cloned from stump-produced "mature-like" cuttings, providing that the material is collected at a suitable stage of development.

In a number of species juvenile shoots are not readily available and thus pretreatments using exogenous hormone applications of BA have been used (THORPE, HARRY and KUMAR,1991; JONES and VAN STADEN,1997). Rejuvenation pretreatments allow for the initiation of *in vitro* cultures of selected shoot cuttings at a period when desirable characteristics are detectable. Rejuvenation also allows for increased multiplication rates, a reduction in hyperhydricity and apical necrosis and allows for improved acclimatisation and field performance (JONES and VAN STADEN,1997). It appears that the greatest degree of rejuvenation may be as a result of using several methods in combination. By recovering juvenile material at the base of the mature plants or through the use of several

rejuvenation methods, it is possible to establish stock plants which will exhibit greater rooting potential and less intracloonal variability than with cuttings taken from the crowns of mature plants. Cuttings of these stock plants would have increased rooting abilities, the desirable juvenile characteristics of vegetative vigour, orthotropic growth and display juvenile branching (HACKETT, 1985). These techniques are often used to activate organs of mature trees prior to *in vitro* culture and are useful pretreatments for further rejuvenation methods *in vitro* (FRANCLET, BOULAY, BEKKAOUI, FOURET, VERSHOORE-MARTOUZET and WALKER, 1987). Rejuvenation methods *in vitro* such as repeated sub-culture of shoot apices on a cytokinin supplemented medium has resulted in an increased endogenous IAA : ABA ratio, resulting in increased rooting. However, plantlets that were produced exhibited plagiotropic growth i.e. partial rejuvenation. For this reason small explants should be used so as to disrupt the controlling forces in the surrounding tissues (THORPE, HARRY and KUMAR, 1991). Even though mature meristems are quite stable *in vivo* and *in vitro*, their phase related characteristics can be changed *in vitro*. Both the length of the culture and number of sub-cultures are related to such changes *in vitro* (HACKETT, 1985). The degree of rejuvenation *in vitro* increases with repeated sub-culture. This was demonstrated on the *in vitro* rejuvenation of *Pinus pinaster* meristems (FRANCLET, BOULAY, BEKKAOUI, FOURET, VERSHOORE-MARTOUZET and WALKER, 1987). It was noted that a high concentration of BA and low concentrations of sucrose, favoured juvenile characteristics. BOULAY (1979) found, when working with *Sequoia sempervirens*, using primary explants of plagiotropic growth, that the orthotropic shoots produced, increased with increased sub-culture. It was also noted that the rooting potential increased with sub-culture (HACKETT, 1985).

In vitro rooting of shoots from mature trees can be difficult. High levels of phenolics and lack of reactivity in mature tissues are the main problems to be solved in order to succeed in the *in vitro* multiplication of adult material (CHAUVIN and SALESSES, 1988). The production of phenolics can lead to necrosis, browning and death. Some suggest culturing explants on a hormone-free medium or lengthy washes in running water. The use of charcoal and a dark treatment also reduces phenolic production. Another factor associated with the *in vitro* culture of adult tissue is hyperhydricity, which leads to various abnormalities (JONES and

VAN STADEN,1997). An important factor to consider when culturing adult tissue is the sterilisation thereof. The ability of *Eucalyptus* to coppice and sprout is advantageous as this provides juvenile material which has reduced contaminants and thus increases sterilisation rates (JONES and VAN STADEN,1997). A hot water treatment has been used in the past for eradication of contaminants, however, this could be detrimental to the plant material concerned (LANGENS-GERRITS, ALBERS and DE KLERK,1998). Cold treatment at 4 ° C for two to four days also reduces internal contamination. Cold treatment has been used with *Lilium* and *Acer*, allowing for mature tissue to be introduced *in vitro* with little difficulty. As with antibiotics, if the contaminant is internal, the contamination may still reoccur at a later stage, especially if the plant material is stressed (KOWALSKI and VAN STADEN,1998).

2.4 The effect of media, plant growth regulators and culture conditions on plant growth

Explant responses in tissue culture are affected by the type of supporting medium, whether it is solid, liquid or semi-solid, whether the explant is cultured in the light or the dark and the inclusion of growth hormones. All these factors contribute to the type of response, positive or negative, and are interrelated in various ways. The type and concentration of gelling agent have an affect on the growth of tissues in culture. The use of low concentrations of gelling agents (BORNMAN and VOGELMANN,1984) or the use of liquid media can result in hyperhydricity and hence the development of the explant (GUPTA, MASCARENHAS and JAGANNATHAN,1981). In Sitka spruce, organogenesis and elongation of shoots was more effective using softer rather than more rigid gels. This was possibly due to the explants being in closer contact with the medium, resulting in better uptake of nutrients and plant growth regulators (WETZSTEIN, CHOONGSIK and SOMMER,1994). BORNMAN and VOGELMANN (1984) noted that the rigidity of agar and Gelrite affected the uptake of BA, with the hormone uptake being inversely related to the gel strength. Lower gel strengths induced more axillary buds from *Picea abies*, however, shooting was counteracted by an increase in hyperhydricity. Agar as a support medium is frequently used, however, it does have a number of negative properties. Agar can inhibit growth, increase hyperhydricity and alter the uptake of cytokinins in the media. When comparing the use of gellan gum (Gelrite) as opposed

to agar as a solidifying agent, there are little differences in BA uptake but due to the impurities present in Gelrite it results in more glassier tissues (BORNMAN and VOGELMANN,1984). TANABE, MURAKAMI, TACHIKAWA, IZUMI, SHIMIZU and MURAKAMI (1995) noted when rooting *Eucalyptus citriodora*, that the use of Gelrite resulted in the leaves turning yellow and abscising. The use of phenol resin foam together with a liquid medium was tested for rooting of *E. citriodora*. Rooting was successful and all rooted shoots survived without subsequent sub-culture, possibly as a result of good aeration. The result was healthy plants which could easily be transferred to the soil as “plug-plantlets” (TANABE, MURAKAMI, TACHIKAWA, IZUMI, SHIMIZU and MURAKAMI, 1995). Hyperhydricity is a common problem in tissue culture and plants with symptoms of hyperhydricity are difficult to multiply and root and often die.

The effects of plant growth regulators on morphogenesis is well documented, however, there are a number of negative side-effects, as noted with high cytokinin levels which have been implicated in promoting hyperhydricity. With *Picea abies*, hyperhydricity increased when explants were cultured for long periods in the presence of BA. A short culture period on optimal BA concentrations followed by a subsequent culture on a hormone-free medium reduced hyperhydricity without affecting budding (BORNMAN and VOGELMANN,1984). TANABE, MURAKAMI, TACHIKAWA, IZUMI, SHIMIZU and MURAKAMI (1995) worked with nodal explants from *Euclayptus globulus*, and noted that the addition of BA stimulated shooting, however, repeated culture on high concentrations of BA inhibited shoot elongation and led to hyperhydricity. The use of high concentrations of cytokinins have been implicated in hyperhydricity in tissue culture (DEBERGH, HARBAOUI and LEMEUR,1981 ; BORNMAN and VOGELMANN,1984). LIEW and TEO (1998) found that production of healthy micropropagated shoots of *Azadirachta excelsa* required an initial culture period with high concentrations of BA and thereafter lower concentrations. The effect of hormones carried over in the plant from the shooting medium, may be detrimental with respect to root initiation. Such affects have been shown with gibberellins resulting in the inhibition of root initials when placed on rooting medium. McCOMB and BENNETT (1986) discovered that the use of gibberellins or the omission of auxin from the multiplication medium, reduced rooting. Similar carry over affects have been found with BA (JONES and VAN STADEN,1997). BON,

BONAL, GOH and MONTEUUIS (1998) when working with *Acacia mangium* and *Paraserianthes falcataria* noted that the addition of plant growth regulators inhibited rooting. Previous work with *A. mangium* showed that cuttings responded well to cytokinins but elongation was problematic. The dominance of the cytokinins may be the cause of the inhibition of rooting (VAN STADEN and HARTY, 1988 ; GALIANA, TIBOK and DUHOUX, 1991). GALIANA, TIBOK and DUHOUX (1991) when working with *A. mangium* saw that plantlets regenerated from juvenile explants had a much lower rooting rate to that of the mature explants. Since BA has a negative affect on rooting, it was deduced that the juvenile tissue must have a high internal cytokinin content or sensitivity. For this reason an initial culture on a low cytokinin concentration (0.5 mg l^{-1} BA) was required for successful multiplication and rooting (GALIANA, TIBOK and DUHOUX, 1991). Shoot elongation is important for the production of plants more adapted for field conditions. Gibberellic acid (GA_3) has been used for elongation and the stimulation of slow-growing cultures, however, extended exposure to GA_3 led to abnormalities and reduced rooting (FRANCLLET and BOULAY, 1982). DAMIANO, CURIR, ESPOSITO and RUFFONI (1989) showed that the use of a rooting "pre-treatment" of low concentrations of NAA, kinetin or zeatin, nullified the Effect of the BA. The use of hormone-free media supplemented with charcoal has been used to induce elongation and enhance rooting in a number of *Eucalyptus* species (JONES and VAN STADEN, 1997). DAS and MITRA (1990) showed that for induction of axial growth from shoot clumps of *Eucalyptus tereticornis*, the hormones in the medium needed to be reduced. The addition of GA_3 and charcoal was stimulatory. A short incubation in the dark helped to stimulate rooting (FURZE and CRESSWELL, 1985 ; DAS and MITRA, 1990). BENNETT, McCOMB, TONKIN and McDAVID (1994) also documented the necessity of a pre-conditioning period of explants prior to rooting. Micropropagation of woody species varies from species to species and within a species. The phase during which an explant may be suitable for rooting also varies. Shoots from mature tissue may require a longer culture period prior to rooting and shoots from coppice may require a shorter period. The detrimental effects of cytokinins (especially BA), with respect to rooting, are well documented (BORNMAN and VOGELMANN, 1984 ; DAS and MITRA, 1990 ; JONES and VAN STADEN, 1997). BENNETT, McCOMB, TONKIN and McDAVID (1994) suggest that BA may stimulate the natural production of cytokinins within

the explant, thus increasing the total available amount of cytokinins, which leads to various abnormalities and reduction in rooting capacity.

The influence of macronutrients can also alter morphogenic responses. BON, BONAL, GOH and MONTEUUIS (1998) documented the influence of a number of macronutrient formulations together with various plant growth regulators on nodal explants of *A. mangium* and *Paraserianthes falcataria*. Almost all morphogenic traits observed for both species were significantly influenced by the various macronutrient media. Half-strength MURASHIGE and SKOOG (1962) medium resulted in better organogenesis than with full-strength MS medium (BON, BONAL, GOH and MONTEUUIS, 1998). Charcoal may promote or inhibit morphogenesis *in vitro*, depending on the species and tissue concerned. The effects of charcoal may be due to it providing a semi-dark environment. Light has been reported to stimulate the reduction of IBA and IAA in both solid and liquid media (NISSAN and SUTTER, 1990), thus charcoal may stabilise plant growth regulators by reducing the light. DUMAS and MONTEUUIS (1995) found that activated charcoal improved *in vitro* rooting of *Pinus pinaster*. as well as the absorption of various hormones and inhibitory compounds (eg. removal of phenolics). The non-selective absorption of charcoal may also result in negative affects (PAN and VAN STADEN, 1998). It is thus extremely important that optimal culture conditions, media and plant growth regulators be used for the plant material which is to be tissue cultured.

2.5 Biochemical indicators of phase transition

In the development of all woody plants, there is a juvenile phase where flowering does not occur and cannot be induced. When a tree is able to flower it is considered to be sexually mature. The length of the juvenile phase is influenced by environmental and genetic factors. This transition is associated with physiological and morphological changes and these changes vary between species. This transition is of importance in the morphological control, differentiation and determination of plant development. It is also significant as the length of the juvenile period is inversely related to the breeding efficiency of the tree and to the selection of improved cultivars (HACKETT, 1987). The decrease or even loss of the ability for true to type cloning as trees age, has been reported for many tree species. The need to find simple markers

of this so-called phase-change phenomenon is important in order to select the more juvenile shoots with greater rooting potential (MONTEUUIS, VALLAURI, POUPARD and CHAUVIE' RE, 1995). The ease of cuttage and *in vitro* propagation is affected by the transitional age. Flowering may indicate that the plant has reached sexual maturity but does not indicate when the transition occurred, as environmental factors may prevent flowering even though the plant may be sexually mature (HACKETT, 1987).

The search for a biochemical marker or cytological marker which is an early indicator of the transition to the mature phase and an intrinsic indicator of differentiation in the cells of juvenile and mature tissues, is an important area of study (HACKETT, 1985). Morphological and chemical markers have been used to identify the most juvenile cells, the best tissue and best time to collect material (BONGA and VON ADERKAS, 1992).

Freshly excised tissues of woody plants, especially angiosperms, often secrete brown pigments consisting of oxidised polyphenols and tannins into the medium, in response to wounding (THORPE, HARRY and KUMAR, 1991). One of the most important characteristics of the Black Wattle is its bark, as this is one of the richest known sources of tannins (ROUX, KEMP and WINGFIELD, 1995). Phenolics inhibit shoot growth and can kill plant material (THORPE, HARRY and KUMAR, 1991). Phenolic production is also problematic in the establishment of cultures *in vitro* (JONES and VAN STADEN, 1997). Explants from more mature tissues have increased levels of phenolics (THORPE, HARRY and KUMAR, 1991), thus tannin production may be an indicator of the transition from juvenility to maturity.

Some authors suggest using a hormone-free medium initially to reduce phenolic production. Others suggest lengthy washing in running water (JONES and VAN STADEN, 1997). However, the most effective method to reduce the amount of phenolics present would be to repeatedly change the culture medium. This may be a lengthy and costly procedure. However, this repeated sub-culture not only reduces the phenolics present it also increases rejuvenation of the explant (THORPE, HARRY and KUMAR, 1991).

2.6 Contaminant eradication through meristem culture

Wattle trees are often infected with various diseases, some of which have led to serious losses in the field. The potential threat of new diseases is of concern, especially as the wattle is planted in monoculture with a relatively uniform genetic base. Exotics have been introduced to a number of diseases to which they lack natural resistance (ROUX, KEMP and WINGFIELD, 1995). An important problem in the propagation of superior tree types is the sterilization of mature tissues. The ability of a mature tree to coppice and sprout reduces the difficulty of sterilization, as the juvenile tissue is less exposed to contaminants (HARTNEY, 1980). Contamination is reduced in both juvenile and meristematic tissues. The apical meristem not only allows for rejuvenated growth, it also allows for the production of contaminant-free plants with reduced levels of other infecting pathogens. This could be extremely beneficial to the wattle industry. Pathogens are difficult to eradicate as they are usually integrally embedded in the plant. Many pathogens are more readily transmitted through cloning than through sexual propagation (BONGA and VON ADERKAS, 1992). The distribution of pathogens, especially viruses within a plant is not uniform and this uneven distribution is the basis for *in vitro* eradication. In 1952 the first virus-free *Dahlia* plants were produced from apical meristem cultures (MOREL and MARTIN, 1952). Viruses and other pathogens are often absent in the terminal meristems and the vegetative apex (BONGA and VON ADERKAS, 1992). It was proposed that the amount of viral particles decreased as the apical dome is reached. Thus the smaller the explant the greater the chance of obtaining pathogen-free plants (NEHRA and KARTHA, 1994). The size of the explant is critical to the success of the procedure. Most workers use shoot tips approximately 0.5 to 1.0 mm in length (DODDS and ROBERTS, 1985). Pathogen removal by meristem culture is best if the leaf primordia are absent. However, it is difficult to establish such cultures from small explants. Larger explants, however, have an increased level of contamination (BONGA and VON ADERKAS, 1992). The host-pathogen relationship is important as this theory of using the apical meristem to obtain pathogen-free plants is not true for all plants. In some plants infected with viruses, the viruses are replicated and transmitted directly to the apical meristem. This depends on the virus and the host (NEHRA and KARTHA, 1994).

Improved results (with respect to the production of pathogen-free plants) have been obtained, usually when meristem culture is combined with heat treatment (INGRAM, 1980). Exposure of the plant to high temperatures prior to the excision of the meristem (BONGA and VON ADERKAS, 1992) or alternatively the culture of meristems at high temperatures also reduced the amount of contaminants present (DODDS and ROBERTS, 1985). Thermotherapy also allows for larger explants to be used. The temperature used depends on the plant and pathogen involved (NEHRA and KARTHA, 1994). At this elevated temperature various pathogens are inactivated yet allowing the plant tissue to continue to grow (DODDS and ROBERTS, 1985).

When tissue culture is used, it is only necessary for a single healthy plant to be produced which is pathogen-free, as this can be further propagated vegetatively. Meristem tips may be regenerated into plants more rapidly than other tissues from other sources and the regenerated plants usually maintain the genetic characteristics of the host plant (WALKERY, 1980). With any pathogen eradication programme, it is important to select a clone which is known to be vigorous and to propagate rapidly, as clones freed from contaminants may in fact be less vigorous than infected clones. It is also important to know which pathogens are present and the extent of meristem infection. The culture medium is also important as it must allow for vigorous, rapid growth (WALKERY, 1980).

Meristem culture has been widely applied because it allows for rapid clonal propagation, virus elimination, germplasm storage and genetic transformation. The shoot apical meristem, seen as a dome of totipotent cells at the tip of the shoot, is the centre of activity for higher plants. The apical meristem undergoes a number of vegetative and reproductive phases. In the vegetative phase, just below the apical meristem are several layers of leaf primordia (NEHRA and KARTHA, 1994). The apical meristem refers only to the region of the shoot apex lying distal to the youngest leaf primordia, whereas the shoot apex refers to the apical meristem and a few leaf primordia (DODDS and ROBERTS, 1985). The history of meristem culture first began when meristems of *Nasturtium* were cultured and rooted plantlets were produced. Since then many advances in this field of research have been achieved (NEHRA and KARTHA, 1994). Meristem culture differs from species to species with respect to the necessary requirements. In 1949 it was shown that fern meristems could be cultured on a simple medium whereas

angiosperms required a more complex medium. The greatest success has been achieved with buds in the juvenile phase of growth or from rejuvenated shoots. Buds taken from mature trees in the adult phase have little capacity for micropropagation. Shoot-apex culture of woody plants requires a number of treatments. Firstly a cytokinin application is required, after which the explants must be transferred to a hormone-free medium to stimulate stem elongation. A third medium including an auxin must then be used to promote root production (DODDS and ROBERTS, 1985).

The second advantage of meristem culture is the mass propagation of clonal material of vegetatively propagated plant species. Although plant regeneration is usually greater by organogenesis and somatic embryogenesis, meristem and shoot-tip culture (which allows for shoot proliferation through axillary branching) is still preferred as this method is less prone to genetic instability (NEHRA and KARTHA, 1994).

2.7 Acclimatisation of regenerated plantlets (*in vivo* and *in vitro*)

Acclimatisation is the final but necessary step in all plant micropropagation programmes. During this period the plants need to adapt to their new environmental conditions. The quality of the plant that is produced *in vitro* is important in governing the success rate during the transition to *ex vitro* conditions. The effectiveness of *ex vitro* establishment determines the success of a micropropagation system. Plants with well established root systems have greater chances of adapting and surviving the acclimatisation stresses. It has also been suggested that once roots are produced the plant should be transferred immediately to potting media to reduce root damage (JONES and VAN STADEN, 1997). Excessive water loss by transpiration and inefficient photosynthetic capabilities are two important problems which must be taken into consideration during the hardening off process (VAN HUYLENBROECK and DEBERGH, 1996). Hardening off often requires a gradual adaptation, where the nutrient medium is simplified and the light intensity increased. Water stress is often a problem thus high humidity conditions must be provided (BONGA, 1977).

SKOLMEN and MAPES (1976) devised a four step procedure for successful hardening off. The plants on agar medium were transferred to Hoaglands solution in flasks and covered with

polyethylene for one month. These were then transplanted to a mixture of peatmoss, perlite and vermiculite and were fertilized monthly with liquid fertilizer and covered with polyethylene for four to six weeks, after transplanting in the laboratory. These were then transferred to the greenhouse after two to three months for further growth, with 70 % full sunlight and increasing temperatures. After one to two months of rapid growth in the greenhouse, plants were moved to an outdoor nursery for two months, prior to field planting (BROWN and SOMMER, 1982).

In *Eucalyptus*, elongated shoots or rooted plantlets were aseptically transferred into a sterile substrate of vermiculite moistened with half-strength MS medium (2:1 v/v) in speedling trays, which were placed in plastic bags to maintain the relative humidity above 70 %. These were maintained for seven days at 25 ° C in a growth room with a 16 hour photoperiod. Thereafter a gradual reduction in humidity was enforced. During this time plantlets were sprayed with a Benlate (0.01 %, Benomyl) / Kelpak (0.1 %, cytokinins and auxins) solution, every alternative day. Plastic bags were removed and plantlets were placed in the greenhouse under mist irrigation. Six to eight weeks later the plantlets were repotted into a soil : sand (2:1) mixture (MACRAE, 1994). DARUS (1991 b) showed that shoots from a micropropagated *A. mangium* x *A. auriculiformis*, which were treated with Seradix (no.3) and planted in a misted rooting chamber containing unsterilized sand, resulted in the production of rooted plants. Vermiculite and soil (1:1) and peatmoss and soil (1:1) are common media and have been used in the acclimatisation process (DARUS, 1991b ; BADJI, MAIRONE, NDIAYE, MERLIN, DANTHU, NEVILLE and COLONNA, 1993). DEWAN, NANDA, and GUPTA, (1992) acclimatised *A. nilotica* using a combination of sand : soil (1:1) together with a high level of humidity. BADJI, MAIRONE, NDIAYE, MERLIN, DANTHU, NEVILLE, and COLONNA (1993) when working with *A. senegal*, used a mixture of vermiculite and peat (1:1) as a potting medium and planted the plants in the greenhouse under a double shade enclosure (provided by a mini-greenhouse together with a canopy of plastic material) at a temperature of 35 ° C for four days. After this the plastic was removed and the plants were mist-sprayed three times for six minutes every 24 hours. The plants were then removed from the greenhouse and misted at 35 ± 8 ° C, under the same watering regimes (BADJI, MAIRONE, NDIAYE, MERLIN, DANTHU, NEVILLE, and COLONNA, 1993).

In vitro plantlets are often mixotrophic or heterotrophic, although carbon assimilation can be stimulated by an increase in light intensity and carbon dioxide concentrations. During acclimatisation an autotrophic metabolism should be developed. Light intensity is also important as *in vitro* grown plantlets are normally grown under a low photosynthetic photon flux density, thus a sudden increase in light intensity will lead to photoinhibition and severe stress. Higher carbon dioxide levels can have a positive effect on the acclimatisation of plants (VAN HUYLENBROECK and DEBERGH, 1996). Experiments conducted on unrooted shoots of *Spathiphyllum* and *in vitro* rooted *Calathea* plantlets, showed that high sucrose concentrations *in vitro* benefit acclimatisation, as starch is accumulated as a nutrient and energy source. However, it was noted that sucrose was used as the main carbon source. Once a functional photosynthetic apparatus was established, there was no difference between sugar and starch metabolism in mixotrophic or autotrophic starting material (VAN HUYLENBROECK and DEBERGH, 1996). In several plant species, an increase in enzymatic activities occur in response to stress situations, which can generate activated oxygen species. Directly after transfer to the greenhouse, plants start to develop an active scavenging system, which is continued during subsequent growth. Features associated with woody species, such as ethylene production, phenol production and slow growth, can be overcome by either increasing the iron-chelates, using antioxidants or by altering the salt composition (RUFFONI, MASSABO, CONSTANTINO, ARENA, DAMIANO, 1991). Gellan gum (Gelrite) or phenol resin foam as a solidifying agent has been used with the result being plantlets in the form of a plug. These “plug” plantlets were then easily transferred to pots of vermiculite and successfully acclimatised (TANABE, MURAKAMI, TACHIKAWA, IZUMI, SHIMIZU and MURAKAMI, 1995). A fibrous supporting medium was also used by IMELDA, KUBOTA, KOZAI and HASEGAWA (1998) in the rooting and successful acclimatisation of *A. mangium* shoots. In their study the use of a fibrous supporting medium together with the absence of sucrose, increased the photosynthetic rate and rooting of *A. mangium* plantlets (IMELDA, KUBOTA, KOZAI and HASEGAWA (1998) .

The adaptation of *in vitro* generated plants to *ex vitro* conditions is a continuous process which requires sufficient time (VAN HUYLENBROECK and DEBERGH, 1996).

CHAPTER 3

PLANTLET REGENERATION FROM *IN VITRO* GROWN EXPLANTS

3.1 INTRODUCTION

Clonal propagation through tissue culture offers an alternative to vegetative practices used in the past and has the potential to provide high multiplication rates of uniform genotypes (GUPTA, PULLMAN, TIMMIS, KREITINGER, CARLSON, GROB and WELTY, 1993). Vegetative practices such as grafting and cuttings have been used in the past for *Acacia mearnsii* with little success. Progress in trying to produce improved seed have been slow due to the wattle plant's long rotation age, and vegetative propagation has been limited due to the plant's poor rooting ability. Today export of wattle timber and by-products thereof constitute a large financial input to the economy of South Africa. Thus, the importance of producing improved seed and maintaining a high level of productivity is relevant.

Previously various degrees of *in vitro* plantlet regeneration, have been achieved with a number of *Acacia* species. However, little work and success have been reported for *A. mearnsii*. GONG, AL-KHAYRI and HUANG (1991) showed callus production on hypocotyl explants. HUANG, AL-KHAYRI and GBUR (1994) micropropagated *A. mearnsii* from shoot tips originating from three-week-old *in vitro* grown seedlings. No further research has been published. It was decided to attempt to regenerate plants using tissue culture techniques, such as organogenesis. Organogenesis is the removal of organs or explants from plant material and culturing them with the hope of producing shoots, roots and ultimately plantlets (DODDS and ROBERTS, 1985).

Acclimatisation is the final but an essential step in all plant micropropagation programmes. During this period the plants need to adapt to their new environmental conditions. The quality of the plant that is produced *in vitro* is important in governing the success rate during the transition to *ex vitro*

conditions. With respect to this aspect, excessive water loss by transpiration and inefficient photosynthetic capabilities are two important problems which must be overcome (VAN HUYLENBROECK and DEBERGH, 1996). Hardening off often requires a gradual adaptation, where the nutrient medium is simplified and the light intensity increased. Water stress is often a problem, thus high humidity conditions must be provided (BONGA, 1977).

DARUS (1991a) showed that shoots from micropropagated *A. mangium* x *A. auriculiformis* cross, which were treated with Seradix (no.3) and planted in a misted rooting chamber containing unsterilized sand, produced rooted plants. Vermiculite and soil (1:1 v/v) and peatmoss and soil (1:1 v/v) are common media which have been used in the acclimatisation process (DARUS, 1991a; BADJI, MAIRONE, NDIAYE, MERLIN, DANTHU, NEVILLE and COLONNA, 1993). DEWAN, NANDA and GUPTA (1992) acclimatised *A. nilotica* using a combination of sand : soil (1:1 v/v) together with a high level of humidity. BADJI, MAIRONE, NDIAYE, MERLIN, DANTHU, NEVILLE, and COLONNA (1993) working with *A. senegal*, used a mixture of vermiculite and peat (1:1) as a potting medium and planted the plants in a greenhouse under a double shade enclosure (provided by a mini-greenhouse together with a canopy of plastic material) at a temperature of 35 ° C for four days. After this period the plastic was removed and the plants were mist-sprayed three times for six minutes every 24 hours. The plants were then removed from the greenhouse and misted at 35 ± 8 ° C, under the same watering regime (BADJI, MAIRONE, NDIAYE, MERLIN, DANTHU, NEVILLE, and COLONNA, 1993). Thus the adaptation of *in vitro* generated plants to *ex vitro* conditions is a continuous process which requires sufficient time and optimal conditions for the species concerned (VAN HUYLENBROECK and DEBERGH, 1996).

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Seeds were sterilized by agitation for ten minutes in off-boiling water. After the ten minute immersion the seeds were rinsed in 70 % ethanol for one minute on a Laminar Flow Bench, and were then agitated for 20 minutes in 1.75 % sodium hypochlorite (3.5%) containing a few drops

of Tween-20. The seeds were then rinsed thoroughly in sterile distilled water and left for 24 hours. Seeds were placed on full-strength MS medium and cultured in growth rooms with a light regime of 16 hours light ($18 \mu\text{mol m}^{-2} \text{s}^{-1}$) / 8 hours dark at a temperature of 25°C .

3.2.2 Explant source, media and supplements

After 30 days, *in vitro* grown plantlets (Figure 3.1 A) were sectioned into root, internodal and nodal sections and then placed onto media containing hormones. Cotyledons were isolated from seeds and used as explants. Explants were placed on MURASHIGE and SKOOG (1962) (MS) medium supplemented with 3 % sucrose, 100 mg l^{-1} myo-inositol and various hormone combinations and concentrations. The pH of the medium was adjusted to 5.8 using 1N HCl or 0.75 M NaOH. The medium was then solidified with 8 g l^{-1} Difco Bacter Agar prior to autoclaving at a temperature of 121°C and a pressure of 103 kPa, for 20 minutes. Four by four hormone grids were established for each experiment and twenty replicates were used per explant. In each experiment the control consisted of MS medium with no hormone supplementation. Explants were incubated under continuous light ($23 \mu\text{mol m}^{-2} \text{s}^{-1}$) or a 16/ 8 hour light/dark photoperiod ($18 \mu\text{mol m}^{-2} \text{s}^{-1}$). The light source for both regimes was provided by cool white fluorescent tubes. The temperature in the growth rooms was maintained at $25 \pm 2^\circ \text{C}$. Table 3.1 shows the auxin and cytokinin combinations which were used. Table 3.2 lists the hormone concentrations that were used.

The various responses of the explants to the respective plant growth regulators, were expressed as a percentage of the number of explants used. Each experiment was repeated twice and the results were averaged. A one-way analysis of variance (ANOVA), with a confidence level of 95 %, was applied to the data.

From these experiments the best explant for shoot and root production was identified and the best hormone combination and concentration for shoot and root production determined. Once these criteria were fulfilled multiple shoot production was initiated and the shoots were then subjected to rooting treatments. Rooted shoots were subsequently used for acclimatisation experiments.

Table 3.1 Auxin and cytokinin combinations used for experiments conducted with *in vitro* grown *A. mearnsii* explants.

Experiment	Cytokinin	Auxin
i	BA	-
ii	BA	NAA
iii	BA	IBA
iv	BA	2,4-D
v	-	2,4-D
vi	-	NAA
vii	-	IBA

Table 3.2 Hormone concentrations tested for *A. mearnsii* plantlet regeneration.

Hormone	Concentration (mg l ⁻¹)							
	0.5	1.0	1.5	2.0	2.5	2.75	3.0	3.25
Benzyladenine (BA)	0.5	1.0	1.5	2.0	2.5	2.75	3.0	3.25
α -Naphthaleneacetic acid (NAA)	-	0.01	-	0.1	-	1.0	-	-
Indole-3-butyric acid (IBA)	-	0.5	0.75	1.0	1.25	-	1.5	1.75
2,4-Dichlorophenoxyacetic acid (2,4-D)	-	0.5	-	1.0	-	-	1.5	-

3.2.3 Acclimatisation of *in vitro* propagated plantlets

Three experiments were conducted using *in vitro* rooted plantlets.

i) *Plantlets placed in a growth cabinet*

Speedling trays were filled with vermiculite (which had been autoclaved at a temperature of 121 ° C and a pressure of 103 kPa) and the vermiculite was subsequently moistened with half-strength MS medim. *In vitro* germinated seedlings were placed into the trays, covered with plastic bags and kept in the growth cabinet for seven days at a temperature of 25 ° C with a 16 hour photoperiod ($18 \mu\text{mol m}^{-2} \text{s}^{-1}$). Plantlets were taken into the greenhouse where the plastic bags were gradually removed in order to decrease the level of humidity gradually.

ii) *Plantlets soaked in Hoagland's solution*

Plantlets were transferred into flasks containing half-strength Hoagland's solution, covered with a polyethylene bag and kept in a growth cabinet for one week at a temperature of 25 ° C with a 16 hour photoperiod ($18 \mu\text{mol m}^{-2} \text{s}^{-1}$). Thereafter plantlets were transplanted into speedling trays containing a mixture of peatmoss : perlite : vermiculite (1:1:1 v/v/v) and moistened with half-strength MS medium. Plantlets were then covered with polyethylene bags and treated monthly with a spray-drench of Kelpak, a seaweed concentrate (1:500 v/v). The plastic bags were gradually removed in order to slowly decrease the level of humidity.

iii) *Plantlets placed in a transparent container under greenhouse conditions*

Rooted plantlets were placed into pots containing perlite and bark chips (1:1 v/v). The pots were totally enclosed in a transparent plastic container (30 x 30 x 15 cm). The containers were placed in a greenhouse at 25 ° C, with a light intensity ranging from 626 to 920 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and were sprayed once a week with Kelpak, a seaweed concentrate (1:500 v/v). After 20 days the plants were exposed to greenhouse conditions for approximately two hours a day for seven days, thereafter they were removed from the plastic container for four hours a day for a further seven days. The plants, which were supported on a table, were then placed on K-cloth and covered with a transparent plastic container. The cloth was kept moist and plants were watered every second day. Plants were kept under these conditions for 14 days thereafter they

were transplanted into pots containing fine bark chips which had been rinsed in 1 % Benlate and placed under the lid of the container (without the bottom of the container). After 14 days the lid was removed and plants were watered every third day.

Plants were kept in the greenhouse until they were sufficiently hardened off to be introduced into the field. The daily temperature in the greenhouse ranged from 20 to 30 ° C in summer and 7 to 25 ° C in winter. The light intensity in the greenhouse ranged from 626 to 920 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

3.3 RESULTS AND DISCUSSION

3.3.1 Optimisation of shoot production

From the results obtained with the various explants from 30-day-old *in vitro* grown seedlings the nodal explant was best for maximum shoot production. It was evident that maximum shoot production occurred in the experiment using BA with concentrations ranging between 2.0 mg l⁻¹ to 3.0 mg l⁻¹. Nodal explants were subsequently subjected to various concentrations of BA (ranging from 0.5 mg l⁻¹ to 3.25 mg l⁻¹) in order to find the optimal cytokinin concentration for maximum shoot production. From Figure 3.2 it is evident that maximum shoot production occurred at a concentration of 2.0 mg l⁻¹ BA, where an average of two shoots per node were produced and 95 % of the explants produced shoots (Figure 3.1 B). A one-way ANOVA was applied. Although shoot production at 2.0 mg l⁻¹ BA was not significantly different from that at 0.5 and 1.5 mg l⁻¹ BA, it was significantly better than with all the other hormone concentrations tested. As this BA concentration resulted in maximum shoot production, it was chosen as the most suitable concentration to use for optimal shoot production. Only the results for the nodal explants subjected to BA concentrations have been presented. Organogenesis from the other explant sources tested irrespective of the hormone treatment, were not significant (Tables 3.1 and 3.2).

3.3.2 Optimisation of root production

The best explant for root production was the internode. Rooting was achieved in experiments using IBA, NAA or 2,4-D. A greater percentage explants produced roots and a greater

number of roots were produced with experiments where IBA was used, thus only these results have been presented. Optimisation of root production was conducted on shoots generated *in vitro*, where concentrations of IBA, ranging from 0.75 to 1.75 mg l⁻¹ IBA were tested. A concentration of 1.0 mg l⁻¹ IBA was the optimal concentration for root production (Figure 3.3) with an average of two roots per shoot and 75 % of the explants producing roots (Figure 3.1 C). A one-way ANOVA was applied and it was apparent that root production at 1.0 mg l⁻¹ IBA was significantly better than the other concentrations tested.

3.3.3 Acclimatisation of *in vitro* propagated plantlets

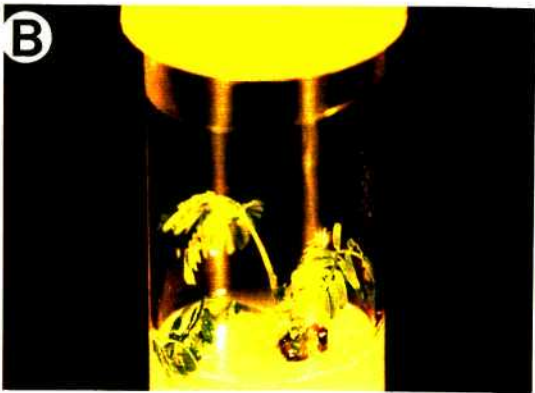
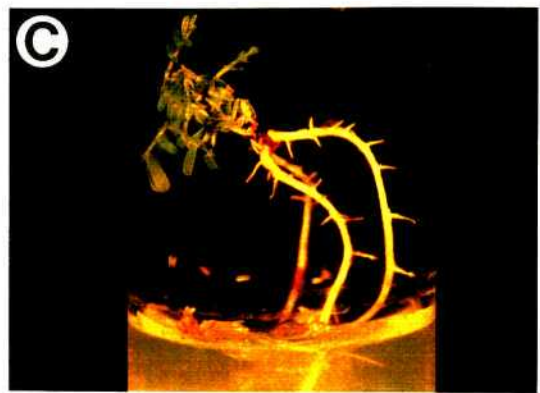
Acclimatisation is the final but important step in all plant micropropagation programmes. In terms of productivity and the relevance of tissue culture, it is important that *in vitro* generated plants be hardened-off and introduced into the field. It is for this reason that many factors must be taken into consideration e.g. light, water, nutrients and humidity. An important factor which determines plant survival is the constituents of the medium they are potted into. The medium must allow for sufficient aeration yet at the same time maintain moisture. It is also important that the acclimatisation process be gradual, allowing the plant to adapt from *in vitro* conditions to *ex vitro* conditions. This is important as it is during this process that the plants develop a cuticle and photosynthesize more efficiently. Thus, the adaptation of *in vitro* generated plants to *ex vitro* conditions is a continuous process which requires sufficient time (VAN HUYLENBROECK and DEBERGH, 1996). Using the above information as a guideline a number of experiments were conducted on *in vitro* germinated seedlings.

i) *Plantlets placed in a growth cabinet*

After one week fungus was noted at the base of the plants. This ultimately led to their death.

ii) *Plantlets soaked in Hoagland's solution*

After one week they were contaminated with fungus and subsequently died.



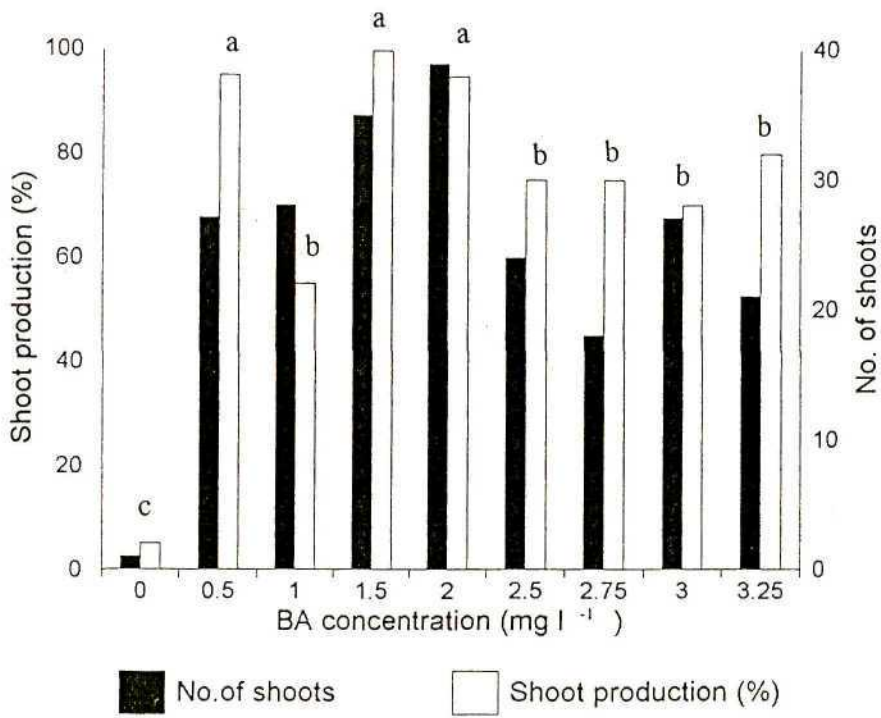


Figure 3.2 Shoot production on *in vitro* grown nodal explants from *A. mearnsii* seedlings using various concentrations of BA. Treatments denoted by the same letters are not significantly different at the 0.05 % level.

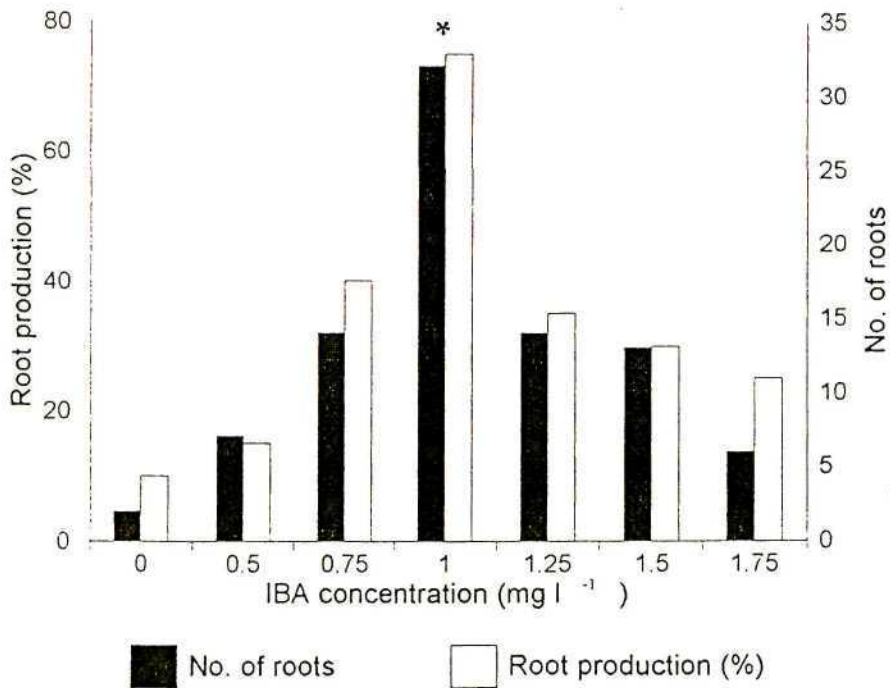


Figure 3.3 Root production from *in vitro* generated *A. mearnsii* shoots using various concentrations of IBA. Treatments, within a tree age, denoted by (*) indicate significant differences at the 0.05 % level.

iii) *Plantlets placed in a transparent plastic container under greenhouse conditions*

In this experiment the plants were enclosed in a transparent plastic container with a sealed bottom. The reason for using a plastic container was to allow light to enter for photosynthesis, yet at the same time maintain a moist, humid environment. The plants were then gradually exposed to air, by initially placing them on K-cloth and eventually removing the K-cloth (Figure 3.1 D). This method of acclimatisation resulted in 90 % survival after a period of 42 days under greenhouse conditions.

3.4 CONCLUSIONS

The best explant for shoot production was the node. Shoot production was achieved on MS medium supplemented with 2.0 mg l^{-1} BA where 95 % of the explants produced shoots with an average of two shoots per node. *In vitro* generated shoots rooted best on MS medium supplemented with 1.0 mg l^{-1} IBA, where 75 % of the explants produced roots with an average of two roots per shoot. With respect to acclimatisation, best results were obtained when plants were initially placed in a closed environment and the humidity then gradually reduced over a period of 42 days. Experiments were conducted where these plantlets were successfully introduced into the field and then compared with seed grown plants and plants regenerated from cuttings (see Chapter 4). Thus the *in vitro* regeneration and acclimatisation of plantlets was successful. This method of plantlet regeneration may be initially more costly than regular vegetative practices, however, on a long term basis the success rate is higher and the entire process is far quicker allowing for a greater turnover rate.

This method also allows for genetically identical material (i.e. clonal material) to be reproduced in bulk, eliminating problems such as cross-pollination, which could result in genetic differences. Thus the results obtained may prove to be extremely beneficial for the wattle industry, in establishing accelerated breeding programmes.

CHAPTER 4

PLANTLET REGENERATION FROM *IN VIVO* GROWN EXPLANTS

4.1 INTRODUCTION

When working with *in vivo* generated plants as explant material, it is necessary to consider suitable sterilization procedures. Prevention and avoidance of contamination is crucial in successful plant tissue culture. Contaminated plants may show no visible symptoms yet may have reduced multiplication and rooting rates or may even die (REED and TANPRASERT, 1995). In order to obtain aseptic cultures it is essential to monitor explants and cultures for contaminants, identify the source and type of contaminant, and subsequently eliminate the contaminating organism with improved cultural practices, antibiotics and / or other chemical agents. Bacterial contaminants are usually difficult to detect as they remain mostly within the plant tissue. Bacterial contamination may originate from a number of sources ranging from the explant, soil, irrigation method to ineffective sterilization techniques. Contaminants of greenhouse-grown plants are normally associated with the soil and may originate from irrigation methods. Epiphytic bacteria may lodge in plant tissues where they are inaccessible to disinfectants (REED and TANPRASERT, 1995). To overcome this problem, surfactants such as Tween-20 are frequently used (DODDS and ROBERTS, 1985). Frequently endophytic bacteria may be found within the plant at cell junctions and in intercellular spaces. Endophytic bacterial contamination is an important problem in tissue culture as it can not be eliminated with surface sterilization techniques and may require antibiotic treatment (REED and TANPRASERT, 1995). Another method to reduce contamination, is to subject plant material to a frequent spraying regime in the greenhouse or garden, prior to taking explants for sterilization (HARTMANN and KESTER, 1975).

Table 4.1 Overview of successful sterilization techniques and explants used for various *Acacia* species.

Species	Explant used	Sterilant tested	Duration	Reference
<i>A. melanoxylon</i>	mature branches	0.2 % mercuric chloride washed in sterile distilled water	20 minutes	MEYER and VAN STADEN (1987)
<i>Acacia</i> species (species not specified)	mature branches cut into pieces	0.6 % JIK 70 % ethanol 10 % JIK washed in sterile distilled water	30 seconds 15 minutes	RUFFONI et al. (1991)
<i>Acacia</i> species (species not specified)	seven-month-old and 13-month-old bipinnate leaves	70 % industrial methylated spirits 5 % (v/v) JIK washed in sterile distilled water antioxidant solution	one minute five minutes 15 minutes	JONES et al. (1990)
<i>A. nilotica</i>	young stem explants	0.1 % mercuric chloride washed in sterile distilled water	five minutes	MARTHUR and CHANDRA (1983)
<i>A. senegal</i>	four-year-old shoot segments	1 % mercuric chloride + 70 % ethanol 70 % ethanol washed in sterile distilled water	seven minutes 30 seconds	BADJI et al. (1993)

Knowledge of the antibiotic and its effect on the plant and bacteria, is important if contamination is to be eliminated and sterile material is to be obtained (REED and TANPRASERT,1995). Every step in the plant tissue culture procedure should be considered in order to prevent contamination. Characterizing the contaminant to determine the species provides important information about the source and the degree of contamination and how to prevent it (REED and TANPRASERT,1995). Extensive *in vitro* research has been conducted on the sterilization of various *Acacia* species. These are listed in Table 4.1.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Plants which had been germinated and grown in the shade house for three, five and nine-months respectively, were used to provide explants.

4.2.2 Sterilization of vegetative plant material

a) Three-month-old plant material

Plants were removed from shade house conditions and were washed thoroughly under running tap water. All further sterilization procedures were conducted on the laminar flow bench, under aseptic conditions. Stem and branch sections were surface sterilized in 70 % ethanol for one minute and soaked in 0.2 % Benlate for a further five minutes. Two sterilization methods were tested. The first involved the use of 1.3 % NaOCl (plus a few drops of Tween-20) for 10, 20 and 30 minutes. The second, involved the use of 0.1 % HgCl₂ (plus a few drops of Tween-20) for 10, 15 and 20 minutes. Plants were subsequently washed in sterile distilled water and then soaked in 50 mg l⁻¹ ascorbic acid for 15 minutes.

b) Five and nine-month-old plant material

Plants were sprayed for a period of seven days with a systemic and a contact fungicide, 0.2 % Benlate combined with 2.2 g l⁻¹ Dithane M-45 (Mancozeb) and 2 % ORGANOCURE ®. Plants were removed from shade house conditions and were washed thoroughly under running tap water. Plants were then placed at 4 ° C for a period of six days in order to help reduce *in vitro*

contamination (KOWALSKI and VAN STADEN, 1998). All further sterilization procedures were conducted on the laminar flow bench, under aseptic conditions. Stem and branch sections were surface sterilized in 70 % ethanol for one minute and soaked in 0.2 % Benlate combined with 2.2 g l⁻¹ Dithane M-45, 1 % Sporekill ® and 2 % ORGANOCURE ® for a further 10 minutes. Stem and branch sections were then sterilized using 0.2 % HgCl₂ (plus a few drops of Tween-20) for 10, 15 and 20 minutes. Plants were subsequently washed in sterile distilled water and then soaked in 50 mg l⁻¹ ascorbic acid for 15 minutes.

4.2.3 Explant source, media and supplements

Three-month-old *in vivo* grown plants were sectioned into three explants, namely the root, internode and node. Nodal explants were removed from five and nine-month-old *in vivo* grown plants. Explants were sterilized and placed on MS medium supplemented with various auxins and cytokinins. Twenty replicates of each explant were used per treatment. In each experiment the control consisted of MS medium with no hormone supplementation. The media, supplements and culture conditions used were the same as those outlined in Chapter 3, Section 3.2.2.

From these experiments the best explant for shoot and root production was identified. The best hormone combination and concentration for shoot and root production was determined. Once these criteria were fulfilled multiple shoot production was initiated and the shoots were then subjected to rooting experiments. These rooted shoots were then subjected to acclimatisation experiments.

4.2.4 Acclimatisation of *in vitro* generated plants

In vitro rooted plantlets were removed from culture and placed into pots containing perlite and bark chips (1:1 v/v). The pots were enclosed in transparent plastic containers and hardened off stated in Chapter 3 Section 3.2.3.

4.3 RESULTS AND DISCUSSION

4.3.1 Three-month-old plants

a) *Sterilization*

Ninety-five percent sterile material was obtained for all explants using 0.1 % HgCl_2 for a period of 15 minutes (Figure 4.1 A).

b) *Plantlet regeneration*

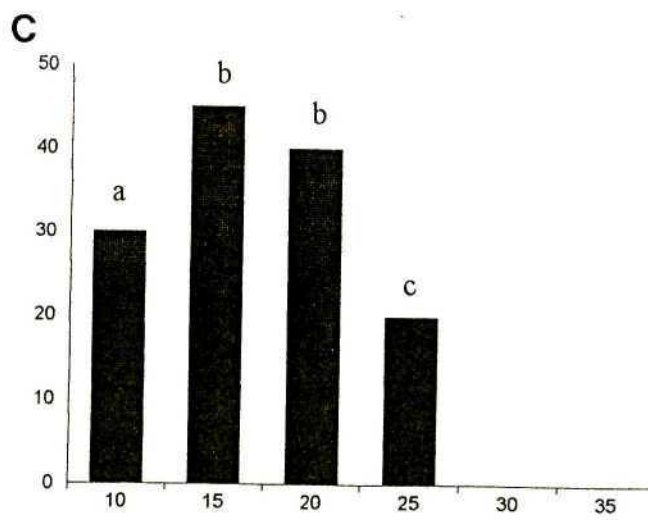
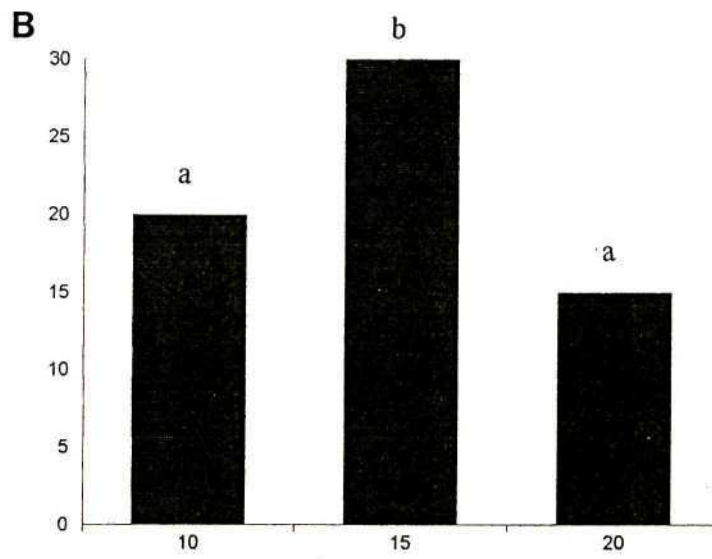
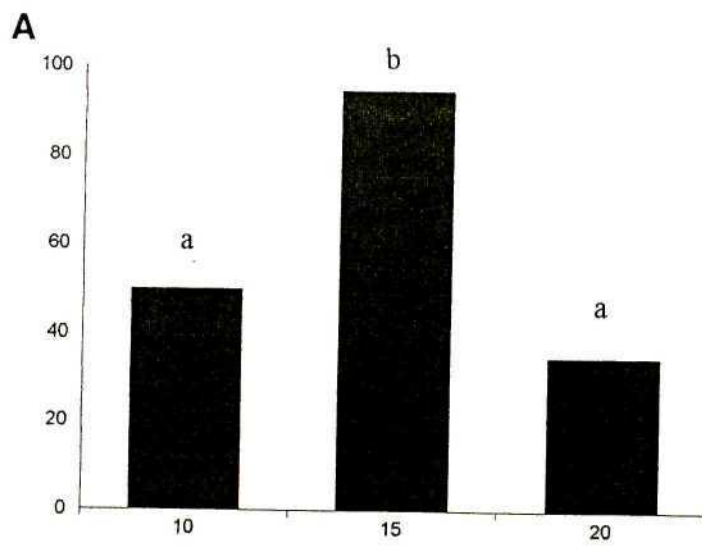
The response of the four explant types tested, varied with respect to the plant growth regulators applied. From the results it was evident that the best explant for shoot production was the node. Maximum shoot production was obtained with BA alone or in combination with NAA or IBA. The best response was at 3.0 mg l^{-1} BA (Figure 4.2) where 25 % of the nodal explants produced shoots (with an average of two shoots per node) (Figure 4.4 A). The best explant for maximum root production was the internodal explant. Optimal rooting was noted at 0.5 mg l^{-1} IBA (Figure 4.3) and at 1.5 mg l^{-1} 2,4-D. These results agree with the results obtained using *in vitro* generated explants (Chapter 3). Only the results concerning shoot and root production with BA and IBA, respectively, were presented as the other data was not significant.

4.3.2 *In vitro* plantlet regeneration using nodal explants from five-month-old plants

a) *Sterilization*

Maximum sterilization was obtained with the 15 minutes treatment using 0.2 % HgCl_2 , where 30 % of the explants were sterile (Figure 4.1 B). Ten and 20 minutes sterilization times gave only 20 % and 15 % sterile material, respectively.

Degree of sterilization (%)



Sterilization time (minutes)

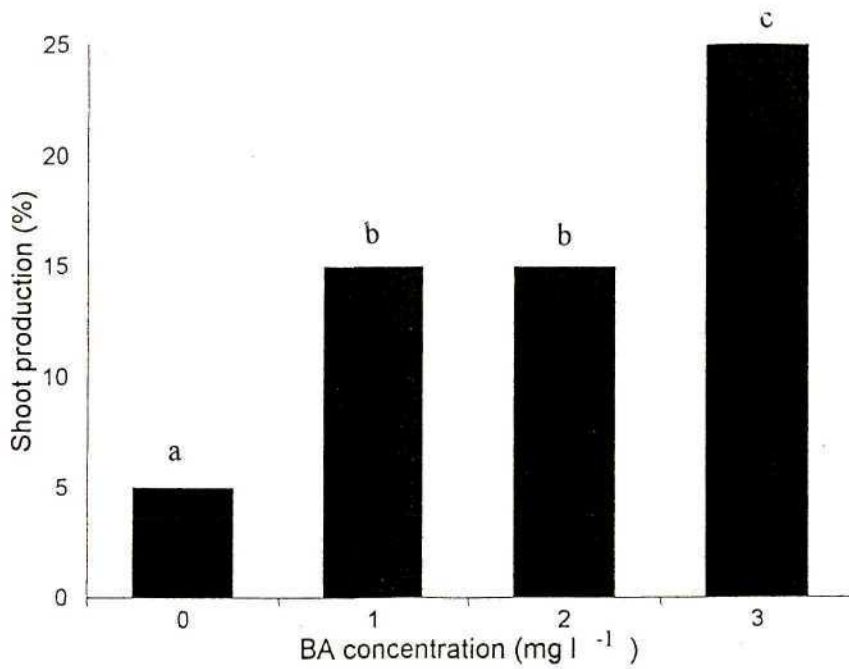


Figure 4.2 Shoot production on nodal material taken from three-month-old *in vivo* grown *A. mearnsii* plants, using MS medium supplemented with various concentrations of BA. Treatments denoted by the same letters are not significantly different at the 0.05 % level.

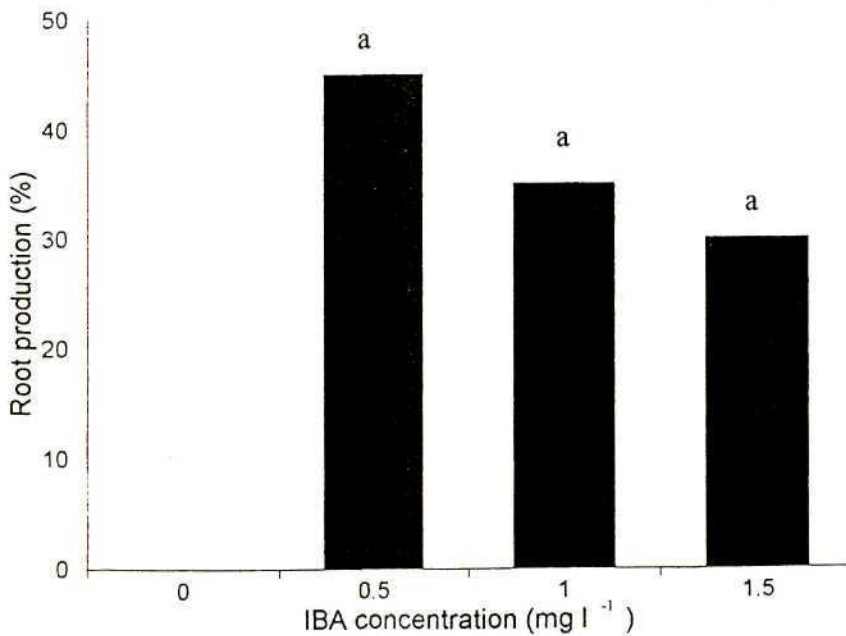
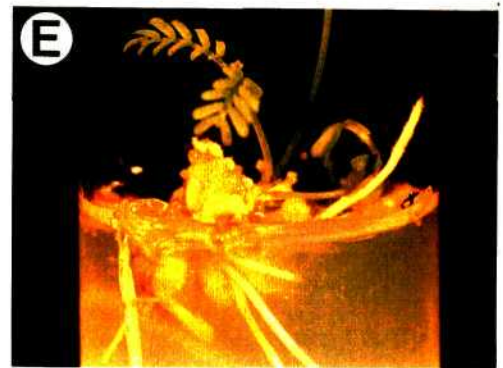
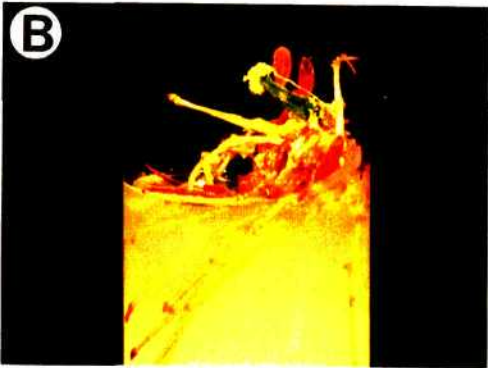


Figure 4.3 Rooting of shoots regenerated on nodes taken from three-month-old *in vivo* grown *A. mearnsii* plants, on MS medium supplemented with various concentrations of IBA. Treatments denoted by the same letters are not significantly different at the 0.05 % level.



b) Plantlet regeneration

Based on the results obtained using explants taken from three-month-old plants and from the results obtained using *in vitro* generated explants (Chapter 3), it was decided that nodal explants be excised and subjected to shooting experiments using a concentration of 2.0 mg l⁻¹ BA. An average of two shoots were produced per node with 15 % of the explants cultured, producing shoots (Figure 4.5 A). Due to the low percentage of sterilized explants (30 % at 15 minutes 0.2 % HgCl₂) (Figure 4.1 B), shoots produced from the sterile nodes were sub-cultured and bulked up (Figure 4.4 C). Once sufficient shoot material was available shoots were rooted on 1.0 mg l⁻¹ IBA. Shortly after being transferred onto rooting media, contamination re-occurred and killed all the plant material.

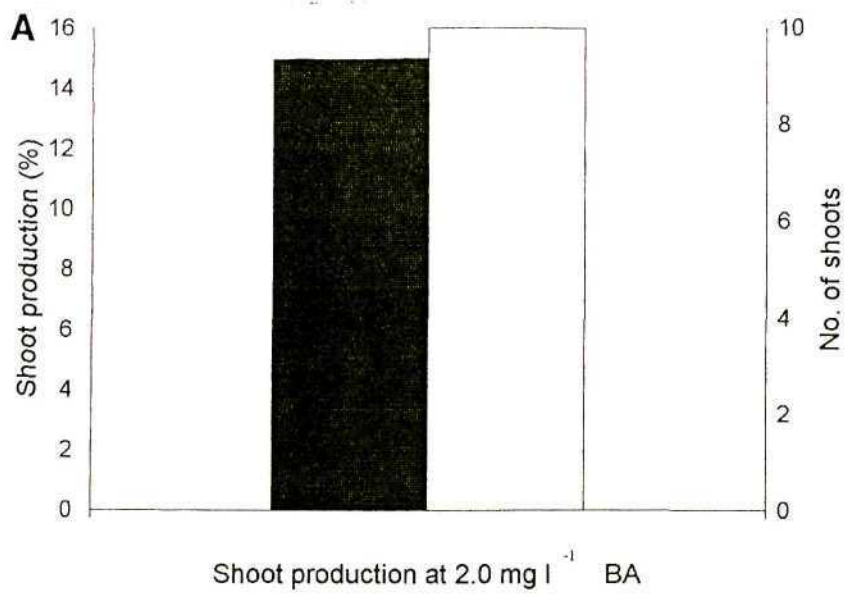
4.3.3 *In vitro* plantlet regeneration using nodal explants from nine-month-old plants

a) Sterilization

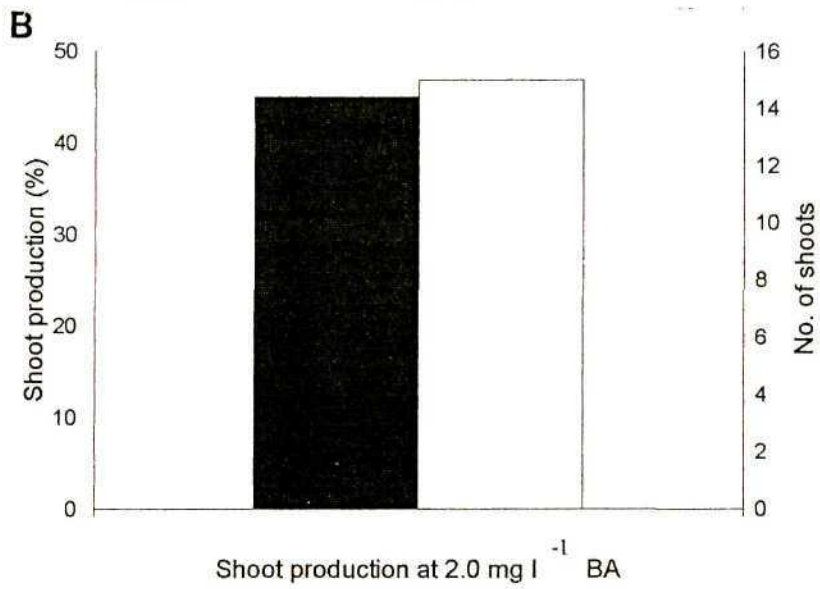
Sterilization periods of 10, 15, 20 and 25 minutes gave 30 % , 40 % , 45 % and 20 % sterilization, respectively. Thus maximum sterilization was obtained by using 0.2 % HgCl₂ for 15 minutes. With this sterilization treatment 45 % of the explants were sterilized (Figure 4.1 C). Using the other sterilization times (10, 20 and 25 minutes) many of the explants were malformed and in poor condition.

b) Plantlet regeneration

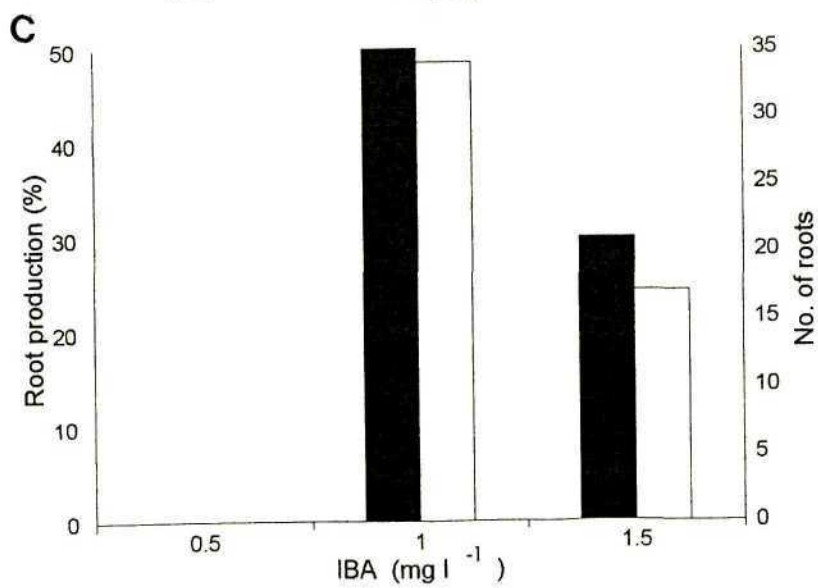
Based on the results obtained using explants taken from three-month-old *in vivo* grown plants and from the results obtained using *in vitro* grown explants (Chapter 3), it was decided that nodal explant be excised and subjected to shooting experiments using MS medium supplemented with 2.0 mg l⁻¹ BA. At this concentration (Figure 4.5 B) an average of one shoot being produced per node and a maximum of 45 % of the explants produced shoots. Due to the small amount of sterile material available, these shoots were sub-cultured until sufficient plant material was available (Figure 4.4 D). These shoots were then excised and rooted on MS medium supplemented with 1.0 mg l⁻¹ IBA (Figure 4.5 C). Fifty percent of the explants produced roots, with an average of three roots per shoot (Figure 4.4 E).



■ Shoot production (%) □ No. of shoots



■ Shoot production (%) □ No. of shoots



■ Root production (%) □ No. of roots

4.3.4 Acclimatisation

Ninety percent of the plants regenerated *in vitro* were successfully acclimatised under greenhouse conditions. Figures 4.6 A to C illustrates plantlets which were regenerated *in vitro*, from nodal explants taken from three-month-old *in vitro* grown plants and from three and nine-month-old *in vivo* grown plants after a period of ten months under greenhouse conditions. At this stage the plastic cover was removed. These plants were kept in the greenhouse for a further five months during the winter and subsequently planted in the field the following summer. A total of 17 plants (three regenerated from seed *in vitro* ; two regenerated from nodal explants taken from 30-day-old *in vitro* grown plants ; six regenerated from nodal explants taken from three-month-old *in vivo* grown plants and six regenerated from nodal explants taken from nine-month-old *in vivo* grown explants) were planted in the field. Plants regenerated from cuttings (from Sunshine Seedlings) were planted together with the tissue-culture generated plantlets (Figures 4.6 D to F). Stick frames were placed around the plants to protect them from Duiker. After 18 months in the field, 14 of the plants survived and were of equal height (approximately 3.5 metres) as those grown from cuttings. The tissue-cultured plantlets had a mortality of 35 %, whereas the plants generated through cuttings had a mortality of 23 %. The tissue-cultured plants had an average stem diameter of 4.4 cm compared to the average stem diameter of the plants generated through cuttings, which was 4.2 cm.

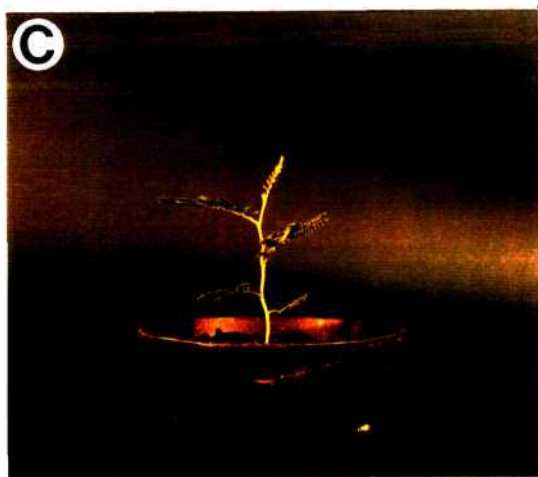
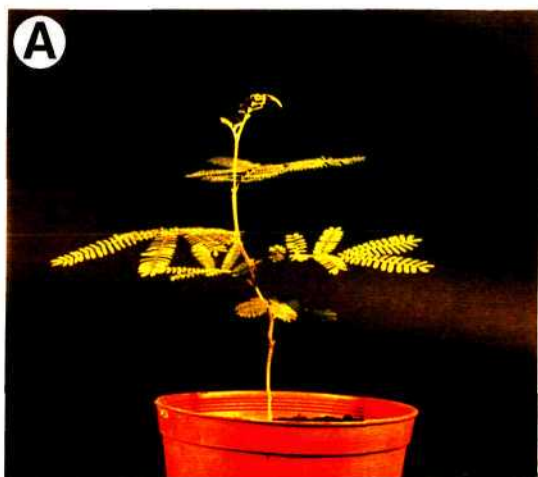
4.4 CONCLUSIONS

The small amount of sterile material obtained for both five and nine-month-old plant material was due to internal contamination of the parent plants. For this reason plants were subjected to a pretreatment with contact and systemic fungicides, Dithane M-45 (Mancozeb) and Benlate (Benomyl) respectively. It was thought that a cold treatment prior to the sterilization procedure might reduce the metabolism of the infecting bacteria making them more susceptible to the sterilant and thus reducing contamination (KOWALSKI and VAN STADEN, 1998). The use of a surfactant Sporekill ® and a treatment of ORGANOCURE ® (a sterilant) all helped to reduce the percentage of contamination, however, the level of contamination increased with tree age. The results obtained using explants from three-month-old *in vivo*

grown plant material were similar to those obtained using *in vitro* generated plant material (Chapter 3). Nodal explants taken from three-month-old *in vivo* grown plant material, were cultured on MS medium supplemented with BA (Figure 4.2). This was the most suitable for multiple shoot production. Maximum rooting was obtained from internodal explants taken from three-month-old *in vivo* grown plant material, cultured on MS medium supplemented with 2,4-D or IBA. Based on these results and the results obtained in Chapter 3, nodal explants taken from five and nine-month-old shadehouse grown material were placed on MS medium supplemented with 2.0 mg l⁻¹ BA for shoot induction (Figure 4.5 B). Shoots that were produced were excised and subsequently placed on MS medium supplemented with 1.0 mg l⁻¹ IBA where they were successfully rooted (Figure 4.5 C). These plantlets were then acclimatised under greenhouse conditions and planted in the field alongside plants regenerated from cuttings. After one year in the field only three plants had died. This was due to heavy rains which flooded and drowned the plantlets (Figure 4.6). After 18 months the tissue-culture regenerated plants compared well with plants generated from cuttings. It can thus be concluded that *in vitro* plantlet regeneration and acclimatisation of three, five and nine-month-old plant material was possible, and that the introduction of *ex vitro* material was successful.

The importance of the results obtained in this Chapter, is that they illustrate the successful introduction and culture of *ex vitro* material, under tissue culture conditions. It is thus important to develop a suitable method of sterilization, that will reduce contamination allowing for *in vitro* culture to continue. If these problems can be overcome, as illustrated in the results obtained in this Chapter, then this is an extremely important step if the ultimate aim is the *in vitro* culture of adult tissue. When working with plant material which has internal contaminants, such as *A. mearnsii*, the percentage of sterilized explants is always going to be low. However, if multiple shooting and multiplication and subsequent rooting can be obtained, then plant numbers can be bulked up and the problem of contamination can be overcome. This is important as it suggests that a single sterilized explant is required, and from this multiple plantlets can be generated.

Since it has been shown that juvenile *ex vitro* material can be successfully decontaminated and introduced into culture, the next step is the introduction of adult tissue into tissue culture.



CHAPTER 5

MICROPROPAGATION OF COPPICE MATERIAL GENERATED ON ADULT *ACACIA MEARNSII* PLANTS

5.1 INTRODUCTION

The black wattle (*Acacia mearnsii* de Willd) is an economically important plantation tree. In South Africa it is grown for the production of tannin and high quality pulp (ROUX, KEMP and WINGFIELD, 1995). The majority of forest trees have, in the past, been propagated by seed. The recent large scale cloning of spruces and eucalypts has, however, validated the importance of clonal propagation. Micropropagation systems have been shown to offer a practical means for clonal propagation of superior forest trees. Cloning allows for the immediate and total capture of genetic gain. The effect of age of the plant material restricts this technique (FRANCLET, BOULAY, BEKKAOUI, FOURET, VERSCHOORE-ARTOUZET and WALKER, 1987). Cloning of mature trees is generally preferred over juvenile trees as it is not always possible to determine if the juveniles will have the desired qualities when they mature (BONGA, 1987). Establishment of cultures *in vitro* using mature tissues is problematic. One of the problems is that by the time the trees are old enough for evaluation, they are often recalcitrant to most forms of vegetative reproduction. As it is easier to propagate juvenile tissues (THORPE, HARRY and KUMAR, 1991), rejuvenation and the establishment of juvenile characteristics prior to *in vitro* culture has proved to be important (JONES and VAN STADEN, 1997).

The use of stump shoots and the practice of coppicing are common methods for rejuvenation (THORPE, HARRY and KUMAR, 1991). Stump sprout explants exhibit better stem elongation and a greater rooting ability than shoots regenerated from the crown (BONGA and

VAN ADERKAS,1992). In a number of species juvenile shoots are not readily available and thus pretreatments using exogenous hormone applications have been used to stimulate shoot growth (THORPE, HARRY and KUMAR,1991).

Rejuvenation pretreatments allow for the initiation of *in vitro* cultures of selected explants at a time when desirable characteristics are detectable. Rejuvenation also allows for increased multiplication rates (FRANCLET,1991). Successful *in vitro* culture of mature trees has been established for *Eucalyptus* species and *Dalbergia sissoo* (ECONOMOU and SPANOUDAKI,1988). TANABE, MURAKAMI, TACHIKAWA, IZUMI, SHIMIZU and MURAKAMI (1995) micropropagated two commercially important *Eucalyptus* species *in vitro*. Multiple shoots were obtained from axillary buds of seven-year-old *Eucalyptus citriodora*, cultured on MS medium supplemented with 0.2 mg l⁻¹ BA and 0.25 % Gelrite as a supporting medium. A low BA concentration was used as high concentrations of BA (0.4 and 0.6 mg l⁻¹) increased hyperhydricity. Hyperhydricity is a common problem in micropropagation as it inhibits shoot elongation and rooting (DEBERGH, HARBAOUI and LEMEUR,1981; BORNMAN and VOGELMAN,1984 ; VIEITEZ, CARMEN SAN-JOSE and VIETEZ,1985). *Eucalyptus globulus* was successfully propagated from nodal segments of three to five-year-old trees (TANABE, MURAKAMI, TACHIKAWA, IZUMI, SHIMIZU and MURAKAMI,1995).

In vitro rooting of shoots from mature trees can be difficult. High levels of phenolics and lack of reactivity in mature tissues are the main problems to be solved in order to succeed with the *in vitro* multiplication of adult material (CHAUVIN and SALESSES,1988). An important factor when culturing adult tissue is the sterilisation thereof. The ability of *Eucalyptus* to coppice and sprout is advantageous as this provides juvenile material which has reduced contaminants and thus increases sterilisation rates (JONES and VAN STADEN,1997). Various treatments have been used to eradicate contaminants, however, as with antibiotics, if the contaminant is internal, the contamination may still reoccur at a later stage, especially if the plant material is stressed (KOWALSKI and VAN STADEN,1998).

The micropropagation of *A. mearnsii* from nodal explants has been achieved, as mentioned in Chapter 3. *Ex vitro* nodal material was subsequently introduced into tissue-culture (Chapter 4) and successfully regenerated. Since the black wattle is an important commercial forest tree it would be economically beneficial to the industry if selected mature tissue could be successfully propagated *in vitro*. As *A. mearnsii* plant material ages, so the ease of obtaining sterile material *in vitro*, decreases. Thus this Chapter deals with the use of coppice as an alternative means of effectively introducing adult tissue into culture. The ultimate aim is to use this technique for future clonal programmes, which are urgently needed.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Trees from five different age groups (two, four, six, eight and ten-years-old, respectively) were selected, decapitated at a height of 1.5 m and all wounds sealed with a commercial tree sealer, Tree Seal (Pruning Grade). An individual control (non-felled) tree from each age group was selected in order to compare adult with juvenile material. These experiments were carried out over a period of two years (1997 to 1999). In the 1997/1998 season the trees were felled in October 1997 and coppice was harvested in November of the same year. In the 1998/1999 season the trees were felled in December 1988 and coppice was harvested in February/March 1999.

5.2.2 Pretreatment and sterilization of vegetative plant material

Plant material was pretreated for a period of seven days with 0.2 % Benlate (a systemic fungicide) plus 1.0 % Sporekill, prior to taking explants. Material was surface sterilized in 70 % ethanol for one minute and soaked in 0.2 % Benlate combined with 0.1 % boric acid for a further ten minutes. Decontamination of the explants was undertaken using 0.1 % and 0.2 % HgCl₂ (plus a few drops of Tween-20) for the coppice and adult material, respectively. Decontamination using mercuric chloride was conducted for a period of 15 minutes. Thereafter the explants were washed thoroughly in sterile distilled water and soaked in 50 mg l⁻¹ ascorbic acid for 15 minutes.

5.2.3 Culture conditions

All cultures were maintained at a temperature of 25 ± 2 °C under light with a 16 hour photoperiod ($23 \mu\text{mol m}^{-2} \text{s}^{-1}$). The light source was provided by cool white fluorescent tubes. The various responses of the explants to the respective plant growth regulators, were expressed as a percentage of the number of explants used. A protected LSD using a 95 % level of confidence was used to separate the means for significant effects from the analysis of variance.

5.2.4 *In vitro* nodal culture and shoot production

a) *Initial investigation (1997-1998)*

Nodal explants (from each of the ten trees felled per age group and from the control tree in each age group) were excised and placed on Murashige and Skoog (MURASHIGE and SKOOG, 1962) medium supplemented with 2.0 mg l^{-1} BA and various concentrations of sucrose (10, 15, 20 and 30 g l^{-1}). The media were solidified with 8.0 g l^{-1} UNILAB Agar and 10 ml was poured into culture tubes. Twenty-five replicates were used for each sucrose concentration. The results for each age group were combined and averaged. Shoots derived from the *in vitro* nodal cultures were maintained by sub-culturing on MS medium supplemented with 2.0 mg l^{-1} BA and 30 g l^{-1} sucrose (HUANG, AL-KHAYRI and GBUR, 1994).

b) *Subsequent investigation (1998-1999)*

Coppice was taken from four age groups (five trees from each age group). Nodal explants were then tested against seven media formulations (Table 5.1). Both jars and tubes were used as culture vessels and the use of Gelrite (3.0 g l^{-1}) as apposed to agar was tested, to see if shooting could be improved. Twenty replicates were tested for each medium. The results were combined and averaged. Shoots produced were sub-cultured onto MS medium supplemented with 0.1 mg l^{-1} biotin and 0.1 mg l^{-1} calcium pantothenate solidified with 3.0 g l^{-1} Gelrite.

5.2.5 Root production

Shoots produced from nodal explants were excised and maintained on hormone-free MS medium for a period of two weeks prior to being placed onto rooting medium.

Table 5.1 Media formulations, supplemented with 2.0 mg l⁻¹ BA, used to culture coppice nodal explants obtained from *A. mearnsii* trees of different ages, decapitated to a height of 1.5 m.

Treatment	Basal medium	Supplementation
1	MS	-
2	MS	0.8 g l ⁻¹ L-glutamine (filter sterilized)
3	MS	0.5 % charcoal
4	½ MS	-
5	¼ MS	-
6	WPM	-
7	240	0.8 g l ⁻¹ L-glutamine

Rooting medium consisted of half-strength MS medium supplemented with 1.0 mg l⁻¹ IBA. Media were solidified with 8.0 g l⁻¹ UNILAB Agar and decanted into jars. All cultures were maintained under conditions of low light (0.72 μmol m⁻² s⁻¹) at a temperature of 26 ± 2 ° C, under continuous light supplied by cool white fluorescent tubes.

5.3 RESULTS AND DISCUSSION

5.3.1 Coppice production

Three weeks after decapitation, coppice was produced on the stumps of all trees (Figure 5.1). With older trees the degree of coppice production on the stumps decreased (Figure 5.2). Greatest coppice production was recorded on the two-year-old stumps and the least on the ten-year-old stumps. There was no significant difference in coppice production between the four, six and eight-year-old stumps ($p < 0.05$). Due to the poor coppice production on the ten-year-old tree stumps, results for this age group were not included.

5.3.2 Decontamination of vegetative material

a) *Plant material taken from the non-decapitated trees (control)*

Decontamination of adult material taken from trees of all age groups was unsuccessful, with 100 % contamination.

b) *Coppice material cultured in the 1997/1998 season*

Sixty to eighty percent decontamination was achieved for the coppice from the two-year-old stumps. Coppice from the four-year-old stumps yielded 58 to 68 % sterilization ; coppice from the six and eight-year-old stumps yielded an average 50 % sterilization (Figure 5.3). On average contamination was in the order of 35 % and no more than 10 % of the explants were killed.



A



B



C



D



E

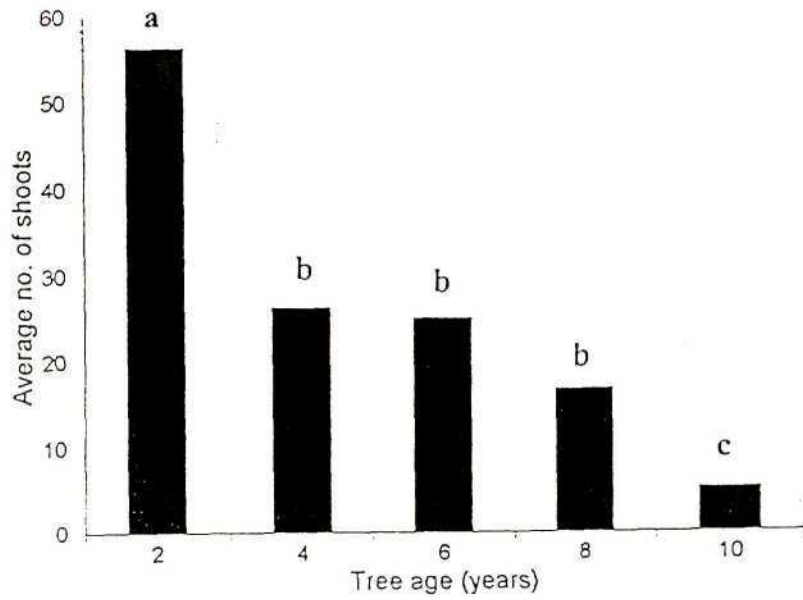


Figure 5.2 Average number of shoots produced on various aged decapitated *A. mearnsii* trees. Treatments denoted by the same letters are not significantly different at the 0.05 % level.

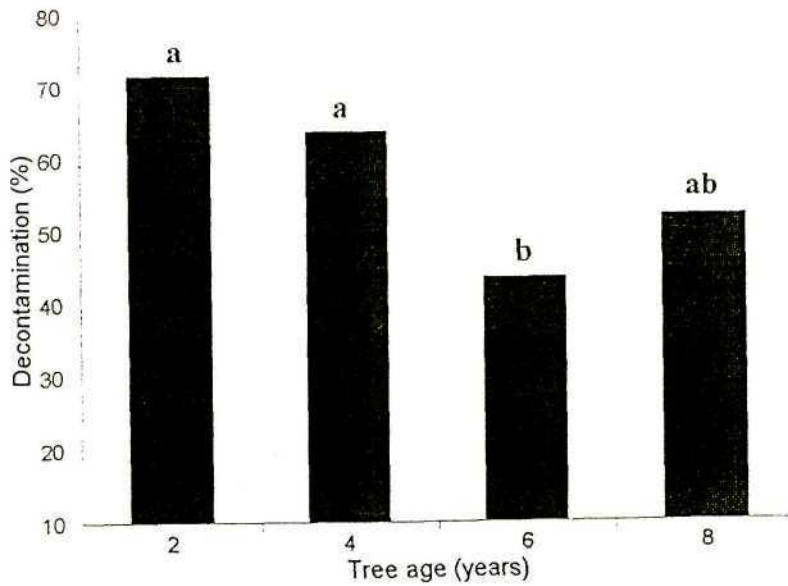


Figure 5.3 Decontamination (%) of coppice nodal explants from coppice material produced by different aged *A. mearnsii* trees decapitated in the 1997/1998 season. Treatments denoted by the same letters are not significantly different at the 0.05 % level.

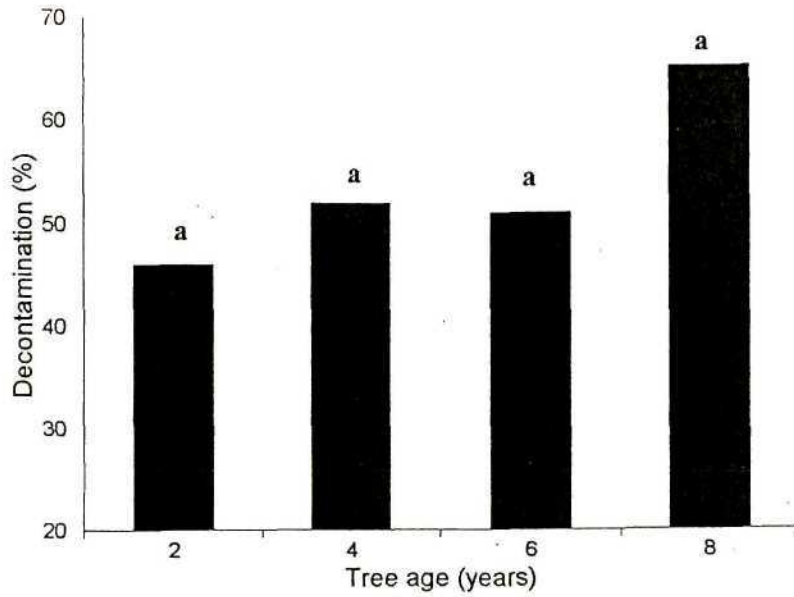


Figure 5.4 Decontamination (%) of coppice nodal explants from coppice material produced by different aged *A. mearnsii* trees decapitated in the 1998/1999 season. Treatments denoted by the same letters are not significantly different at the 0.05 % level.

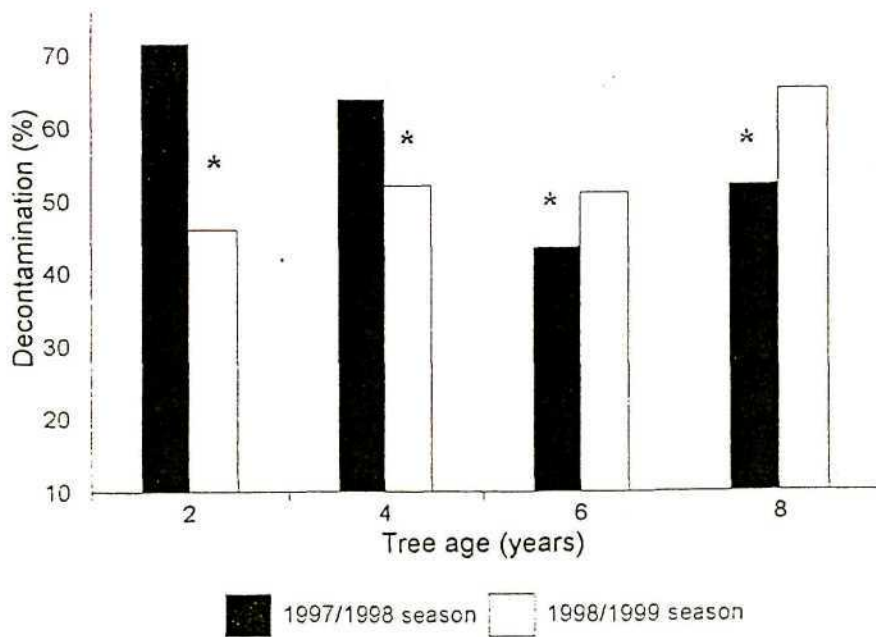


Figure 5.5 Decontamination (%) of coppice nodal explants from four different ages of *A. mearnsii* trees over two seasons (1997 to 1999). Treatments, within a tree age, denoted by (*) indicate significant differences at the 0.05 % level.

The high level of successfully decontaminated explants throughout the tree age is an indication that the method of coppicing reduces contamination thus increasing *in vitro* performance. There was a slight decrease in the amount of sterilized explants obtained as the tree age increased. However, decontamination of adult trees was still high (50 %). This suggests that coppicing might reduce contamination *in vitro*.

c) *Culture of coppice material taken in the 1998/1999 season*

There was no obvious trend indicating a decrease in decontamination as tree age increased (Figure 5.4). A greater percentage of successfully sterilized explants were obtained from coppice produced on the eight-year-old tree stumps than from the other tree ages. However, there was no difference in the percentage sterilized explants obtained from coppice taken all tree ages. The percentage contamination in all tree ages was no greater than 35 % and death of plant material was not more than 15 %. This once again indicates that the use of coppice material reduces contamination *in vitro* and that the age of the tree producing the coppice has no obvious effect on the *in vitro* performance of the coppice nodal explants.

d) *Comparison of decontaminated material obtained from coppice from stumps of four tree ages over the two seasons (1997 to 1998)*

With reference to Figure 5.5, it is evident that the method of producing coppice was successful over both seasons. Seasonal variation will effect the degree of coppice production and physiological state of the coppice. A possible reason for the increase in survival in the 1998/1999 season for coppice taken from the six-year-old tree stumps, could be attributed to contamination. In the 1997/1998 season the six-year-old trees were infected with a worm which reduced coppice production and hence limited the plant material for *in vitro* culture. It is apparent that sterilized coppice material can be produced from all tree ages, regardless of the environmental conditions. However, seasonal affects may affect the amount of sterile material obtained. Temperature remained fairly constant throughout both seasons, ranging from 25 to 27 ° C (Appendix A, Figure 1). Rainfall, however, was greater in the 1997/1998 season, with 93.6 mm compared to 51.8 mm received in the 1998/1999 season. It is possible to concluded that under conditions of high

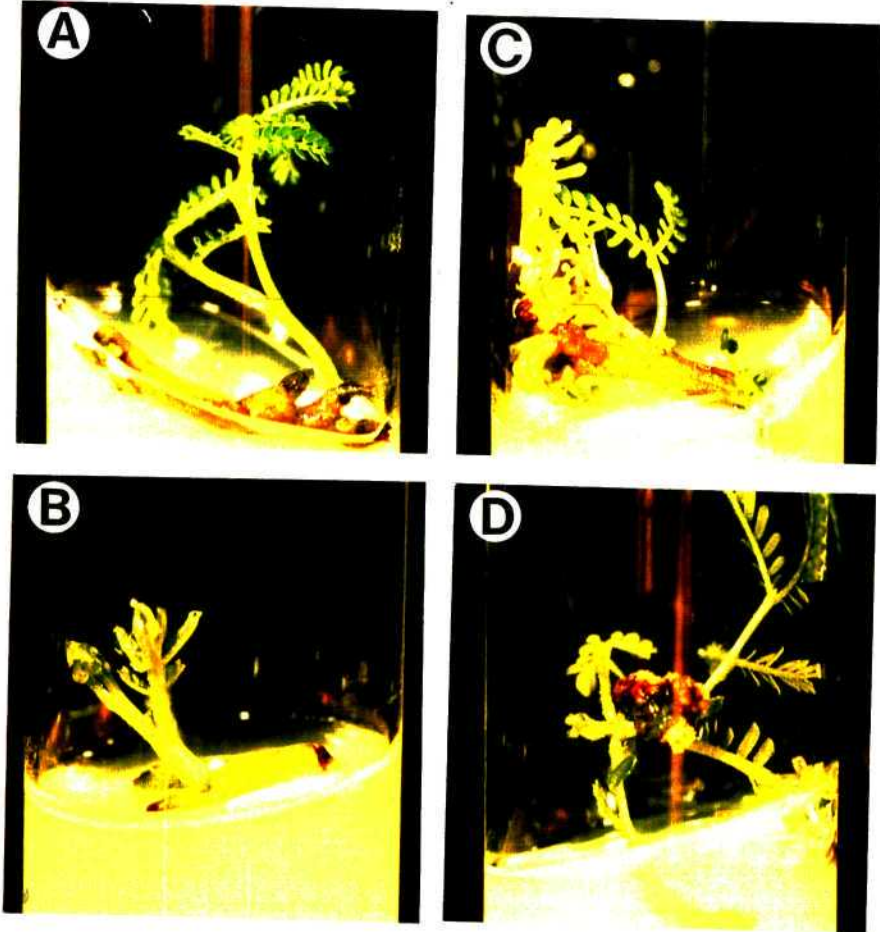
temperature and high rainfall (as in the 1997/1998 season), nodes taken from coppice material has increased chances of survival under tissue culture conditions.

5.3.3 *In vitro* nodal culture

a) *Shoot production from explants placed in the 1997/1998 season*

After a period of approximately 14 days multiple shoot production was obtained from cultured material derived from all tree ages (Figures 5.6 A to E). Sixteen percent of the nodes taken from coppice produced on the two-year-old tree stumps, produced shoots. Twenty-two percent of the nodal explants excised from coppice produced on the four and eight-year-old tree stumps, produced multiple shoots and maximum shooting was obtained from coppice taken from the six-year-old trees (Figure 5.7). Shoot production increased, as the age of the tree stumps from which coppice nodal material was obtained, increased. However, there was no significant difference in shoot production by coppice nodal explants taken from various aged tree stumps ($p < 0.05$). It is interesting to note that the greatest percentage sterile explants were obtained with the coppice taken from the two-year-old tree stumps yet of these explants only a low percentage produced shoots. The least amount of sterilized explants were obtained from coppice produced on the six-year-old tree stumps, however, these explants had the greatest percentage shooting, thus, indicating that a low percentage of sterile explants is not detrimental if the shoot production from the explants is high. This is important especially when dealing with plant material which has internal contamination and the percentage sterile explants obtained will always be low.

Maximum shoot production from coppice nodal explants, taken from various aged trees, occurred at an increased sucrose concentration (30 g l^{-1}) (Figure 5.8). This was statistically significant ($p > 0.05$). This was contrary to reports in the literature which state that *Pinus pinaster* meristems cultured on a high concentration of BA and low concentrations of sucrose, favoured rejuvenation (HACKETT, 1987). However, coppice is considered as juvenile tissue and thus should provide



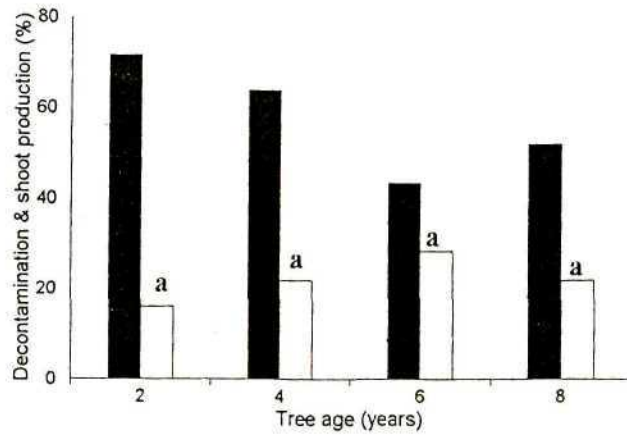


Figure 5.7 Decontamination (%) (■) and shoot production (%) (□) on coppice nodal explants from various aged *A. mearnsii* tree stumps. Results were recorded in the 1997/1998 season, 14 days after sterilization. With respect to shoot production amongst age groups, treatments denoted by the same letters are not significantly different at the 0.05 % level.

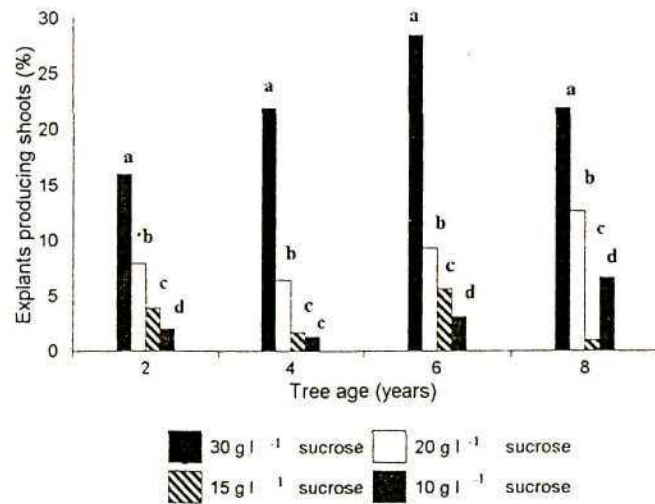


Figure 5.8 Shoot production (%) on coppice nodal explants from various aged *A. mearnsii* tree stumps, on MS medium supplemented with different concentrations of sucrose (g l^{-1}). Results were recorded in the 1997/1998 season, after a period of 14 days. Treatments, within a tree age group, denoted by the same letters are not significantly different at the 0.05 % level.

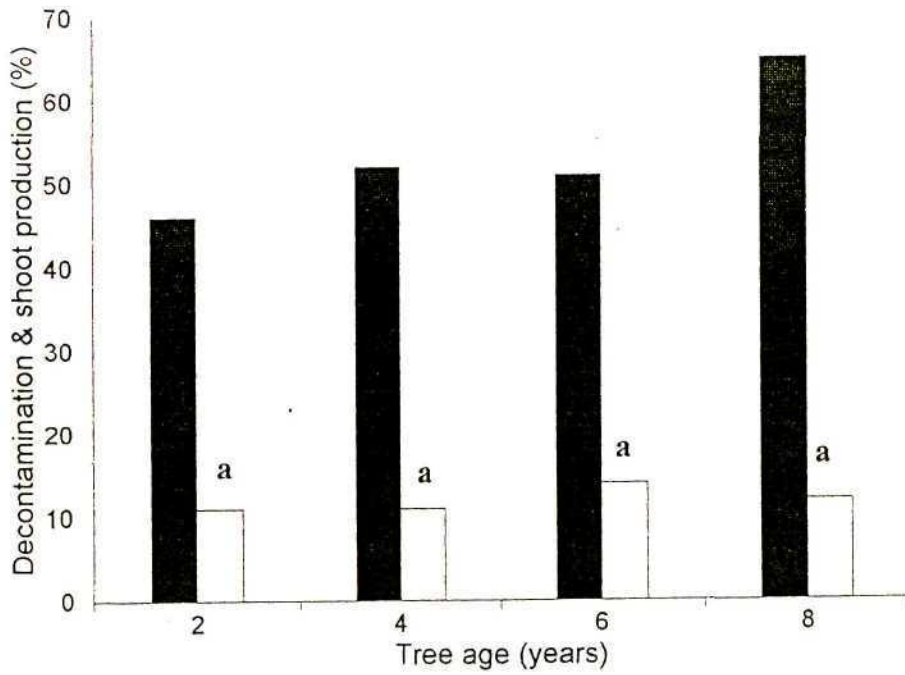


Figure 5.9 Decontamination (%) (■) and shoot production (%) (□) on coppice nodal explants from various aged *A. mearnsii* trees stumps. Results were recorded in the 1998/1999 season. With respect to shoot production within a tree age, treatments denoted by the same letter are not significantly different at the 0.05 % level.

meristematic growth (HACKETT, 1987), hence the need for a higher carbohydrate supply. It has been shown that the practice of coppicing both increased *in vitro* performance and eliminated the effect of maturation. This is in agreement with the literature for other hardwood trees (MONTEUUIS, VALLAURI, POUPARD and CHAUVIERE, 1995).

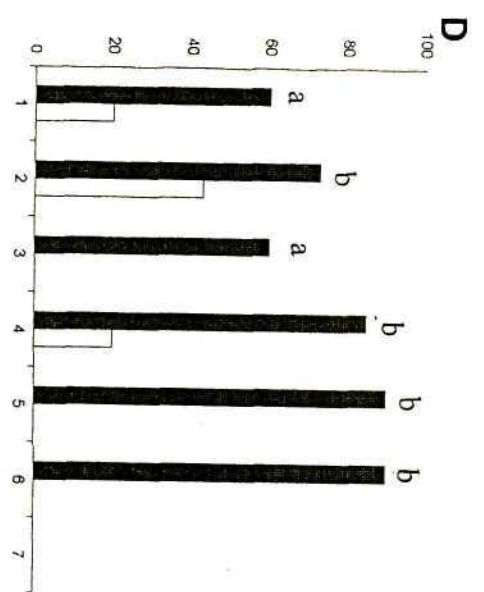
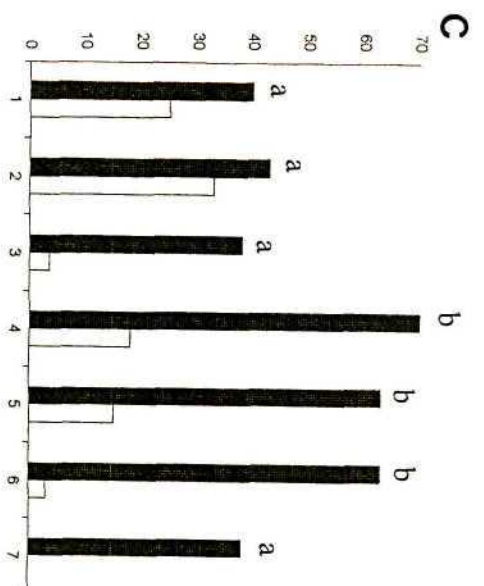
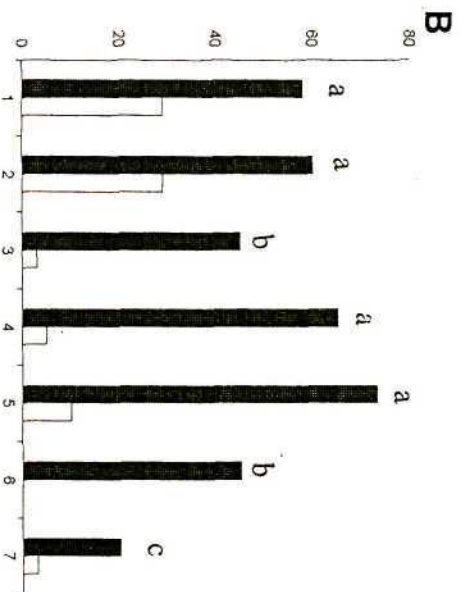
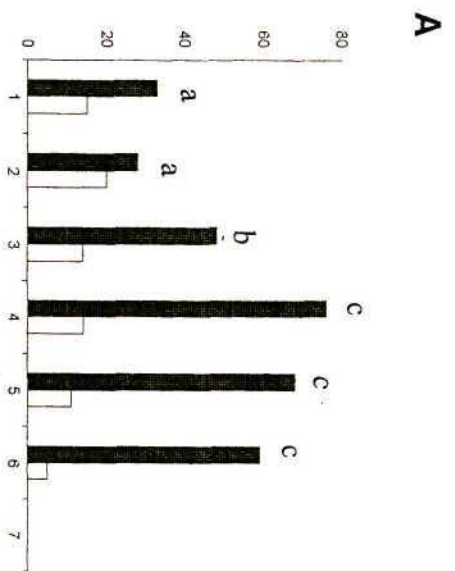
b) Shoot production from explants placed in the 1998/1999 season

Multiple shoot production occurred on nodal explants taken from coppice from all trees. Up to 11 % shooting was obtained from coppice taken from the two, four and eight-year-old tree stumps. Fourteen percent of the nodes produced on the six-year-old tree stumps produced shoots (Figure 5.9). There was no significant difference in percentage shoot production as the age of the tree increased ($p < 0.05$). This was in agreement with the results obtained from the previous season. Thus the use of coppicing in rejuvenating adult material was successful and could become a routine practice. Survival of coppice nodal explants taken from different aged trees, was greatest where the MS basal medium was used at half and quarter strength (treatment 4 and 5, respectively) (Table 5.1) and where Woody Plant Medium was used (treatment 6) (Figure 5.10 A to D). However, maximum shoot production was achieved on treatment 1 and 2. The addition of glutamine in treatment 2 increased shoot production on nodal explants taken from coppice produced on trees stumps of various tree ages.

c) Comparison of percentage shoot production obtained from the 1997/1998 and 1998/1999 seasons

When comparing overall shoot production (Figure 5.11), there was a similar trend for both seasons, where shoot production from nodal explants increased to a maximum with coppice taken from the six-year-old tree stumps and then decreased slightly, but this was not significant ($p < 0.05$). However, in the 1997/1998 season a greater percentage shooting was obtained than in the 1998/1999 season. The differences in shoot production between the two seasons could be due to the fact that different trees were used in each season and also due to seasonal variation. Trees in the 1997/1998 season were decapitated in early October and due to a rainy season coppice production occurred on the stumps after three weeks.

Decontamination and shoot production (%)



Media tested

From the 1997/1998 season four sets of cuttings were taken from the stumps for all age groups. Due to the low rainfall (Appendix A, Figure 1) experienced in February (1999), only one set of cuttings could be obtained. The combination of high temperatures and low rainfall experienced in the 1998/1999 season (Appendix A, Figure 1) would have stressed the plants, explaining the poor performance of the explants both in the field and *in vitro* and reducing the amount of plant material available and the percentage shoot production *in vitro*.

Over the two seasons, the use of 25 ml tubes and 100 ml jars were tested for holding the medium. Gelrite and UNILAB agar were tested as solidifying agents. In some experiments the use of Gelrite was superior as a solidifying agent. The use of Gelrite can be advantageous in mobilising nutrients and making them more accessible to the explants. Agar as a support medium is used frequently, however, it does have a number of negative properties. Agar can inhibit growth, increase hyperhydricity and alter the uptake of cytokinins in the media (DEBERGH, HARBAOUI and LEMEURE, 1981 ; BORNMAN and VOGELMANN, 1984). The use of jars appeared to be advantageous. By using jars there is possibly an increase in available oxygen for the explants, thus enhancing their performance. A future in depth study to compare holding vessels and solidifying agents could be beneficial.

5.3.4 Root induction

a) Shoots produced from the 1997/1998 season

Shoots produced from the coppice nodal material were sub-cultured on MS medium supplemented with 2.0 mg l^{-1} BA in order to bulk up the number of shoots for rooting experiments. Individual shoots were excised from the explant and cultured on MS medium supplemented with 1.0 mg l^{-1} IBA, in the light for rooting. After two weeks, explants were either recontaminated through endogenous bacteria, or defoliated due to stress, or showed signs of hyperhydricity and failed to root. Defoliation is an indication of stress (TANABE, MURAKAMI, TACHIKAWA, IZUMI, SHIMIZU and MURAKAMI, 1995).

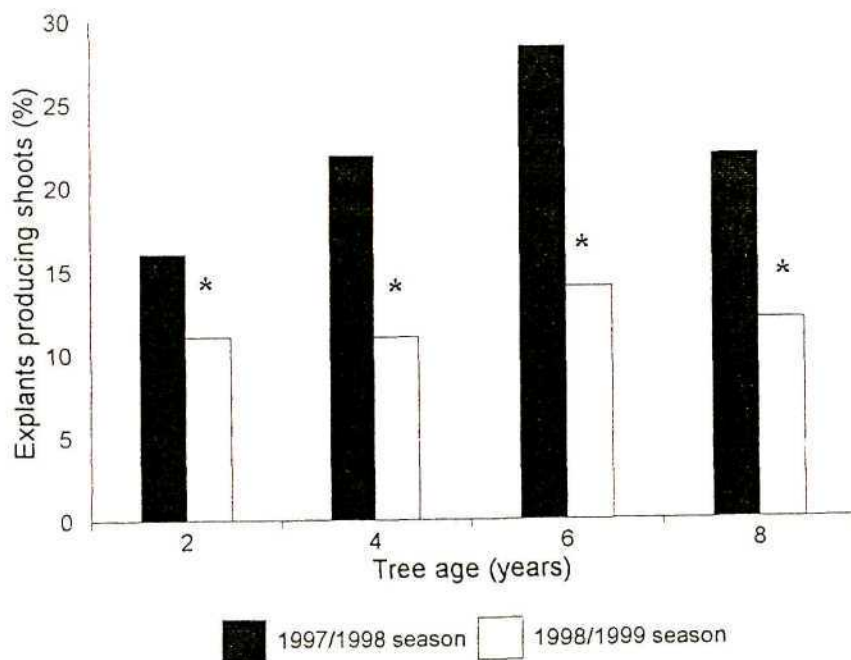


Figure 5.11 Comparison of shoot production (%) on coppice nodal explants from coppice produced on various aged *A. mearnsii* tree stumps, in the two seasons tested (1997 to 1999). Treatments, within a tree age, denoted by (*) are significantly different at the 0.05 % level.

The negative carry-over effects of cytokinins, especially BA, have been widely documented (BORNMAN and VOGELMANN,1984 ; JONES and VAN STADEN,1997). Continuous culture especially BA, have been widely documented (BORNMAN and VOGELMANN,1984 ; JONES and VAN STADEN,1997). Continuous culture of shoots on high concentrations of BA can be detrimental resulting in hyperhydricity and death of the explants (BORNMAN and VOGELMANN,1984 ; LIEW and TEO,1998). It is thus important that shoots be cultured for a period of time on hormone-free medium (JONES and VAN STADEN,1997) or media supplemented with charcoal (PAN and VAN STADEN,1998) to reduce the levels of cytokinins in the explants. When working with IBA the use of charcoal or culture under conditions of low light are recommended to increase rooting (FURZE and CRESSWELL,1985 ; DAS and MITRA,1990 ; PAN and VAN STADEN,1998) possibly by decreasing the rate of IBA degradation by light.

b) Shoots produced from the 1998/1999 season

In view of the results obtained in the previous season, shoots produced on the coppice nodal explants were sub-cultured onto hormone-free MS medium supplemented with 0.1 mg l⁻¹ biotin and 0.1 mg l⁻¹ calcium pantothenate for a period of two weeks. The use of hormone-free medium was to help reduce the levels of cytokinins still present in the explant from the shoot initiation stage, which might impede root production. Thereafter shoots were sub-cultured onto half-strength MS medium supplemented with 1.0 mg l⁻¹ IBA. Cultures were maintained under conditions of low light (0.72 μmol m⁻² s⁻¹). After a period of two weeks, contamination once again reoccurred and no rooting was achieved. In future a longer culture on hormone-free media prior to rooting, should be applied, as it is possible that, cytokinins were still present in the explant and thus hindered root development.

5.4 CONCLUSIONS

Acacia mearnsii reaches maturity in approximately ten years (BEARD,1957), thus progress in attempting to produce improved seed for wattle-growing farmers, has been slow due to the long rotation time and limited by the poor rooting abilities of wattle cuttings. Clonal propagation

through tissue culture offers an alternative to vegetative practices used in the past and has the potential to provide high multiplication rates of uniform genotypes, resulting in short term gains (GUPTA, PULLMAN, TIMMIS, KREITINGER, CARLSON, GROB and WETTY, 1993). Clonal forestry has increased in significance as an alternative to conventional vegetative practices (HAN, SHIN and KEATHLEY, 1997). *In vitro* cloning of mature trees is generally preferred over juvenile tissues as it is not always possible to determine if the juvenile material will have the desired qualities when mature (BONGA, 1987). Establishment of cultures *in vitro* using mature tissue is often problematic (JONES and VAN STADEN, 1997). Successful *in vitro* establishment of explants from adult *Acacia* trees has been achieved with *A. auriculiformis* (WANTANABE, IDE and IKEDA, 1994; REDDY, VEERANA, GOUDA, PRASAD, PADMA, UDAYAKUMAR and PATIL, 1995; TODA, TAJIMA and BRINI, 1995; ZHANG, HUANG, FU, YANG and CHEN, 1995), *A. mangium* (TODA, TAJIMA and BRINI, 1995; ZHANG, HUANG, FU, YANG and CHEN, 1995) and *Robinia pseudoacacia* (HAN, SHIN and KEATHLEY, 1997). In all of the above studies axillary buds were used as explants and the results varied from 28 % shoot production for axillary buds from two-year-old *A. auriculiformis* seedlings (TODA, TAJIMA and BRINI, 1995), to 71.4 % for buds from two-year-old *A. mangium* seedlings (TODA, TAJIMA and BRINI, 1995).

The problem with the propagation of hardwoods, is their difficulty in rooting (a maturation effect). Once the trees are ready for genetic evaluation, they are often recalcitrant to most vegetative practices (JONES and VAN STADEN, 1997). As it is easier to propagate juvenile tissues (THORPE, HARRY and KUMAR, 1991), rejuvenation and the partial re-establishment of juvenile characteristics prior to *in vitro* culture, has proved to be important (JONES and VAN STADEN, 1997). From the results in this Chapter, the use of coppice material as a means of rejuvenating and introducing adult *A. mearnsii* tissue into culture for improved multiple shoot production was possible. Coppicing reduced the levels of contamination *in vitro* and increased juvenile characteristics of the adult material. This allows for mature tissue to be easily propagated *in vitro*. In both seasons a similar trend was noted, where shoot production increased to a maximum with the coppice taken from the six-year-old tree stumps and then decreased slightly.

However, there was no significant differences in the results obtained from coppice nodal explants taken from the tree ages ($p < 0.05$). The detrimental effect of low rainfall combined with high temperatures, on shoot production was evident in the results obtained in the 1998/1999 season. However, shoot production was possible from coppice obtained from trees up to ten-years-old. As noted in the results obtained from both seasons, only a small amount of sterile explants are required, providing that the shoots produced can be multiplied. The reoccurrence of contaminants at later stages due to stress may necessitate the use of an antibiotic. Endogenous contamination is thus still a problem *in vitro* and possibly the use of an antibiotic in the initial sterilization, or later, once the contaminant has been released, would help to increase survival of the explants and increase morphogenic responses

With respect to the *Acacia* species in particular, the use of tissue culture in breeding programmes has only been applied to *A. melanoxylon*, where it was used to provide early amplification of limited seed (HAN, SHIN and KEATHLEY, 1997). Due to the economic importance of the black wattle and the need to establish a successful clonal programme for it, the results obtained from this study are particularly important. The results achieved thus far, indicate that adult material can be rejuvenated through coppicing and coppice nodal material can be decontaminated and stimulated to produce shoots *in vitro*. Thus the black wattle, as with other forest trees, can be micropropagated *in vitro*. The findings reported in this Chapter are promising and will at a later stage play an important role in the development of a successful means of micropropagating the black wattle.

CHAPTER 6

MERISTEM CULTURE OF *ACACIA MEARNSII*

6.1 INTRODUCTION

The shoot apical meristem, seen as a dome of totipotent cells at the tip of the shoot, is the centre of activity for higher plants (NEHRA and KARTHA, 1994). Meristem culture has been widely applied to a number of plant and crop species. It is evident that there is a strong and intricate interaction between the explant, plant growth regulators, culture conditions and genotype. The use of meristem culture for virus elimination has been employed for a number of species (MOREL and MARTIN, 1952). Viruses and other pathogens are often absent in the terminal meristems and the vegetative apex and thus apical meristem culture not only allows for rejuvenated growth, but also for the production of contaminant-free plants with reduced levels of other infecting pathogens (BONGA and VON ADERKAS, 1992).

Wattle trees (*Acacia mearnsii* de Willd) are often infected with various diseases, some lead to serious losses in the field. The potential threat of new diseases is of concern, especially as the wattle is planted in monoculture with a relatively uniform genetic base. As exotics they have been introduced to a number of diseases to which they lack natural resistance (ROUX, KEMP and WINGFIELD, 1995). An important problem in the propagation of superior tree types is the sterilization of mature tissues (JONES and VAN STADEN, 1997). Contamination is reduced in both juvenile and meristematic tissues. Thus increasing the chances of *in vitro* plantlet regeneration. This could be extremely beneficial to the wattle industry.

When meristem culture is used, it is only necessary for a single healthy plant to be produced which is pathogen-free, as this can then be propagated vegetatively. Furthermore, meristem tips may be regenerated into plants more rapidly than tissues from other sources and the regenerated plants usually maintain the genetic characteristics of the host plant (WALKERLY, 1980). The greatest success has been obtained from buds in the juvenile phase

of growth or from rejuvenated shoots. Another advantage of meristem culture is the ability for mass propagation of clonal material of vegetatively propagated plant species. Although organogenesis and somatic embryogenesis are common methods for *in vitro* plant regeneration, meristem and shoot-tip culture (which allows for shoot proliferation through axillary branching) are still preferred as these methods are less prone to genetic instability (NEHRA and KARTHA, 1994). *Pinus pinaster* meristems were successfully cultured and elongated using a basal medium with a high concentration of sucrose and low concentration of BA (HACKETT, 1985). The only reported success of meristem or shoot tip culture in *Acacia* species, has been with *A. saligna*. Shoot tips ranging between two to three centimetres in length were excised and placed on solidified MS medium supplemented with 5.0 to 9.0 mg l⁻¹ BA. Clusters of multiple shoot buds were then placed on MS medium supplemented with 0.3 mg l⁻¹ BA and 0.2 mg l⁻¹ IAA, for elongation. Rooting was achieved on MS medium supplemented with 2.0 mg l⁻¹ IBA (BARAKAT and EL-LAKANY, 1992).

This Chapter reports on the culture of meristems from *in vitro* germinated *Acacia mearnsii* seedlings, field grown plants and coppice tissue.

6.2 MATERIALS AND METHODS

6.2.1 Plant material

Plant material was obtained as mentioned in Chapter 5, section 5.2.1. An individual intact tree from each age group was selected in order to compare adult with juvenile coppice material. Shoots were obtained from branches of these trees and from coppice produced on stumps of the decapitated trees of different ages. These experiments were carried out over a period of two years (1997 to 1999). In the 1997/1998 season the trees were felled in October 1997 and coppice was harvested in February/March 1998. Adult plant material was harvested in March/April 1998. In the 1998/1999 season the trees were felled in December 1998 and coppice was harvested in February/March 1999 and adult plant material was harvested from September to December 1998.

6.2.2 Seed sterilization and *in vitro* germination

Seeds were sterilized as mentioned in Chapter 3, Section 3.2.1.

6.2.3 Pretreatment and sterilization of coppice and adult branch material

Coppice and adult plant material was pretreated and sterilized as mentioned in Chapter 5, Section 5.2.2.

6.2.4 Culture conditions

In the 1997/1998 season all cultures were maintained at a temperature of 25 ± 2 °C. All cultures were placed in the light ($23 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16 hour photoperiod. The light source was provided by cool white fluorescent tubes. In the second season both light ($23 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a 16 hour photoperiod) and dark culture conditions were tested. All cultures were maintained at a temperature of 25 ± 2 °C. The various responses of the explants to the respective plant growth regulators used, were expressed as a percentage of the number of explants excised. Results were combined and averaged and a protected LSD using a 95 % level of confidence was used to separate the means for significant effects from the analysis of variance. Due to the small nature of the explants and the clusters of shoots produced, it was difficult to count individual shoots produced. Results were thus expressed as a percentage of explants that survived and had undergone shoot development i.e. it was noted if the size of the explant had increased and if shoot initials were evident.

6.2.5 *In vitro* meristem culture

a) Initial investigation (1997/1998)

Meristems from *in vitro* grown, adult and coppice material were removed microscopically and placed on MS medium alone. One hundred replicates were used. The results were combined and averaged. Shoots derived from the *in vitro* meristem cultures were maintained by sub-culturing on MS medium with no hormonal supplementation. A UNILAB Agar support medium (8.0 g l^{-1}) was used. Ten millilitres of medium was decanted into 65 mm sterile plastic

petri dishes (Labotec). The size of meristems that were removed ranged from 0.5 to 1.0 mm (Figure 6.1 A).

b) Subsequent investigation (1998/1999)

i) Adult material

Liquid and agar supporting media were tested under both light and dark culture conditions (refer to section 6.2.4). Liquid cultures in 50 ml Erlenmeyer flasks were kept on a continuous shaker at 150 rpm. Seventy replicates (ten replicates of seven meristems per plate) were used per experiment using the agar supporting media. One hundred replicates (ten replicates of ten meristems per 50 ml Erlenmeyer flask) were used for the liquid suspension cultures. The 50 ml Erlenmeyer flasks contained 20 ml medium. Eight treatments were tested (Table 6.1). *Acacia mearnsii* is a leguminous tree and thus it was thought that the addition nitrogen in the form of glutamine, might enhance *in vitro* performance.

ii) Coppice material

Based on the results obtained from the adult material, only an agar-solidified medium was used, for coppice material. Both light and dark culture conditions were investigated. Based on the results obtained from the adult material, only four variations of media were tested (treatment 1, 2, 5 and 7, Table 6.1). The size of meristems that were removed ranged from 1.0 to 2.0 mm (Figure 6.1 B). After the initial production of small clusters of shoots, explants were sub-cultured onto MS media containing two different concentrations of gibberellic acid

Table 6.1 Media formulations tested for organogenic responses from adult and juvenile *A. mearnsii* explants placed during the 1998/1999 growing season. Cultures were placed in 65 mm sterile plastic petri dishes.

Treatment	Basal medium	Plant growth regulators (mg l ⁻¹)	Supplementation
1	MS	-	-
2	MS	-	0.8 g l ⁻¹ L-glutamine (filter sterilized)
3	MS	2.0 BA	-
4	MS	2.0 BA	0.8 g l ⁻¹ L-glutamine
5	½ MS	-	-
6	½ MS	-	0.8 g l ⁻¹ L-glutamine
7	WPM	-	-
8	240	2.0 BA	0.8 g l ⁻¹ L-glutamine

(GA₃) (0.1 and 1.0 mg l⁻¹) and a control consisting of MS medium supplemented with 0.1 mg l⁻¹ biotin and 0.1 mg l⁻¹ calcium pantothenate. The use of GA₃ was to stimulate shoot elongation. The use of both jars and petri dishes was investigated at this stage to determine if an increase in aeration might enhance shoot elongation and production. Forty millilitres of media were decanted into 100 ml culture jars. These cultures were maintained under conditions of low light (0.72 μmol m⁻² s⁻¹) at a temperature of 26 ± 2 °C, and under conditions of light (23 μmol m⁻² s⁻¹) with a 16 hour photoperiod.

6.3 RESULTS AND DISCUSSION

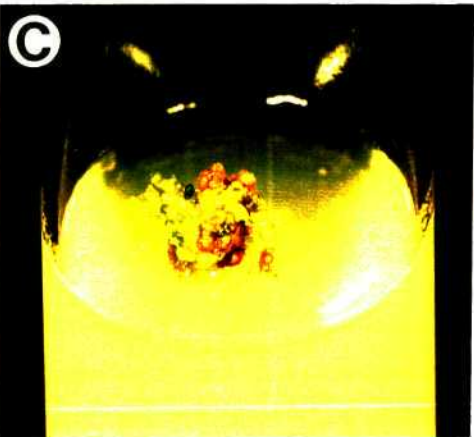
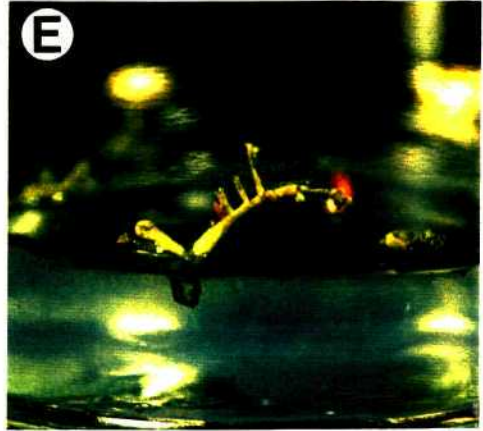
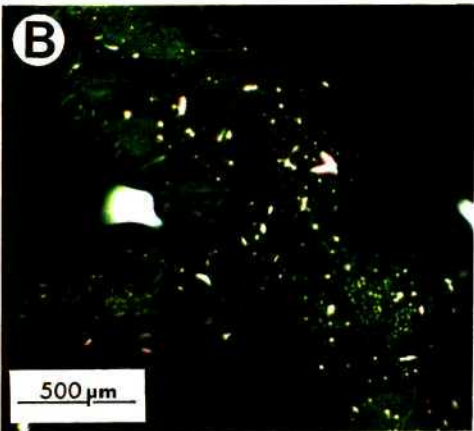
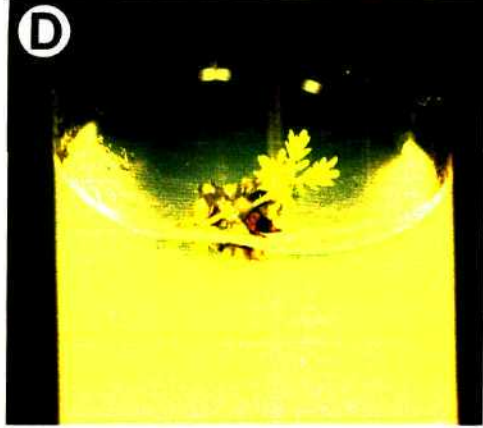
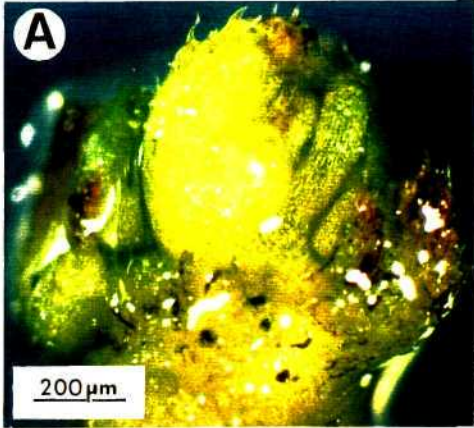
6.3.1 Meristem culture of vegetative plant material collected in the 1997/1998 season

a) *Meristems from in vitro grown plants germinated from seeds*

Seeds were successfully sterilized and within a period of seven days 98 % of the seeds had germinated. After 30 days seedlings had developed *in vitro* and meristems were excised. After a period of three months 40 % produced shoots on MS medium alone (Figure 6.1 C). A large percentage (55 %) of the meristems died due to the small size of the explant. Contamination did not exceed 5 %.

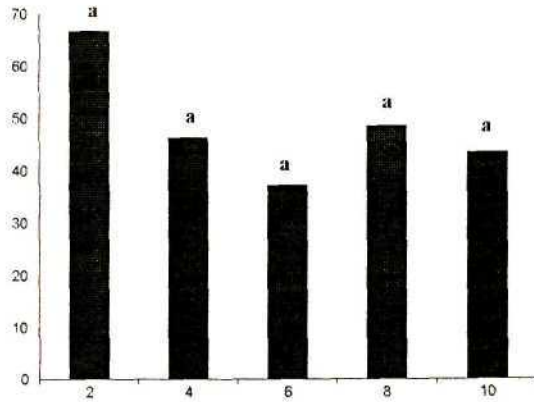
b) *Meristems from adult branches*

Decontamination of apical tips taken from all trees of five ages was successful, with contamination levels being no greater than 3 % (Figure 6.1 D). Shoot production occurred irrespective of the age of the donor trees (Figure 6.2 A). The best shooting was obtained with meristems taken from the two-year-old trees, averaging between 53 and 67 %. Thereafter, as the age of the donor tree increased, shoot production remained unchanged. However, there was no significant difference in shoot production between the meristems taken from all tree ages ($p < 0.05$). This suggests that the age of the host tree was not a limiting factor in plantlet regeneration from meristems taken from adult trees.

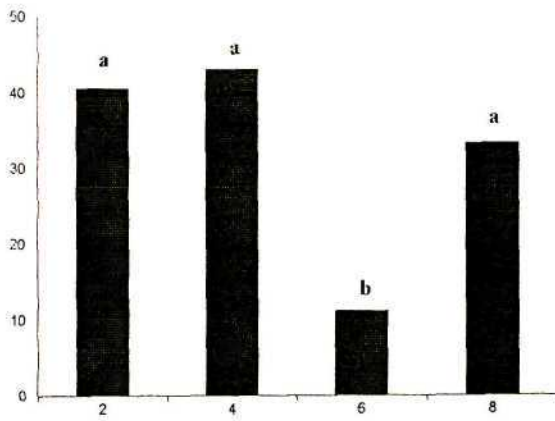


Shoot production (%)

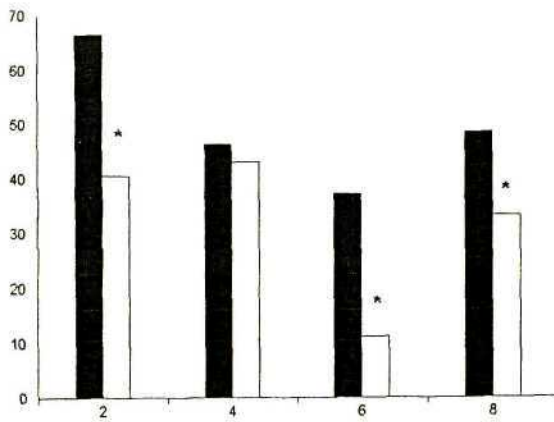
A



B

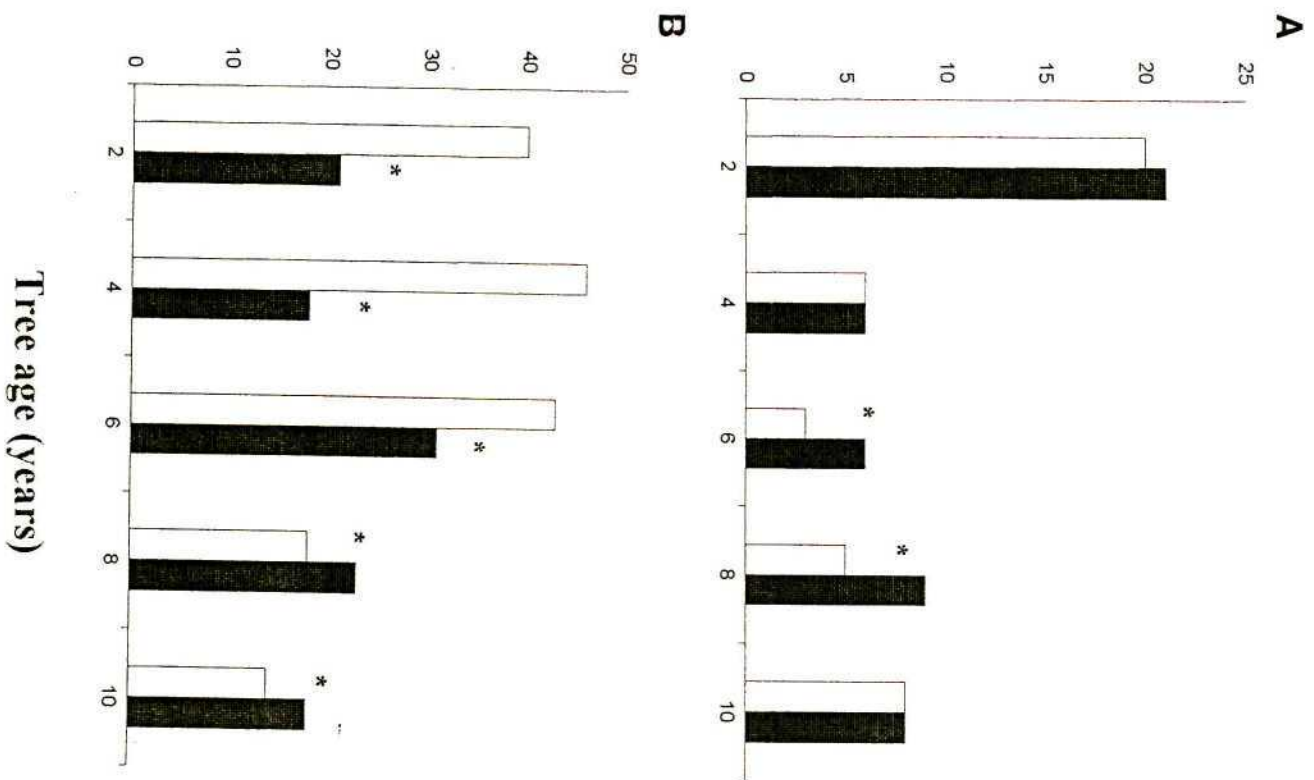


C



Tree age (years)

Contamination (%)



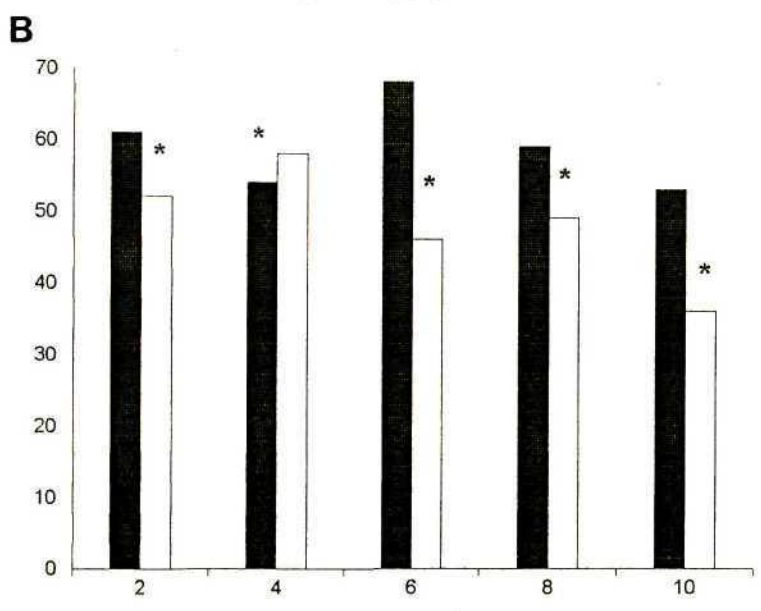
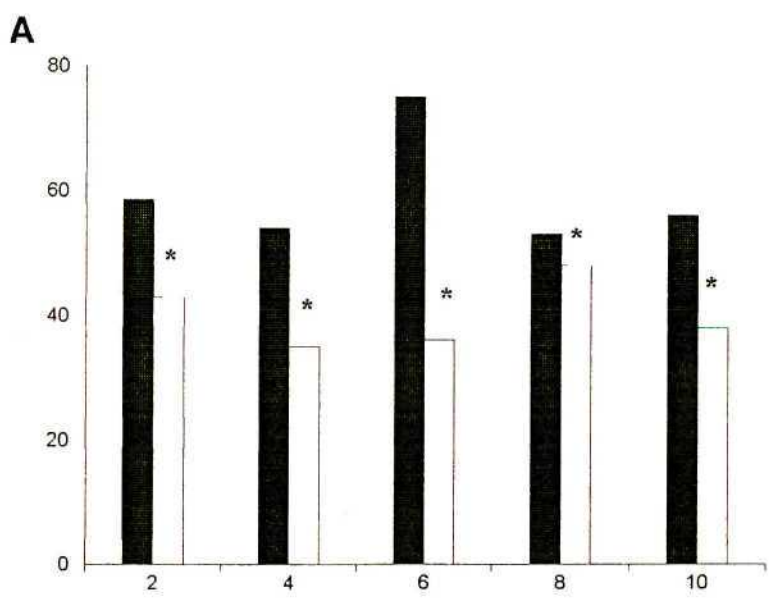
c) *Meristems from coppice material*

Due to the poor coppice production on the ten-year-old tree stumps, results for this age group were not included. Decontamination and shoot production was successful for coppice meristems taken from trees of all four ages. With reference to Figure 6.2 B, there was no significant difference in shoot production from coppice meristems taken from the two, four and eight-year-old trees ($p < 0.05$). Shooting from coppice of this material ranged from 33 to 43 %. Shooting from coppice from six-year-old trees was significantly lower (12 %) ($p > 0.05$) due to an infection in the stumps. There was no distinct trend correlating a decrease in shoot production with tree age.

d) *Comparison of shoot production by meristems from adult and coppice explants from the 1997/1998 season*

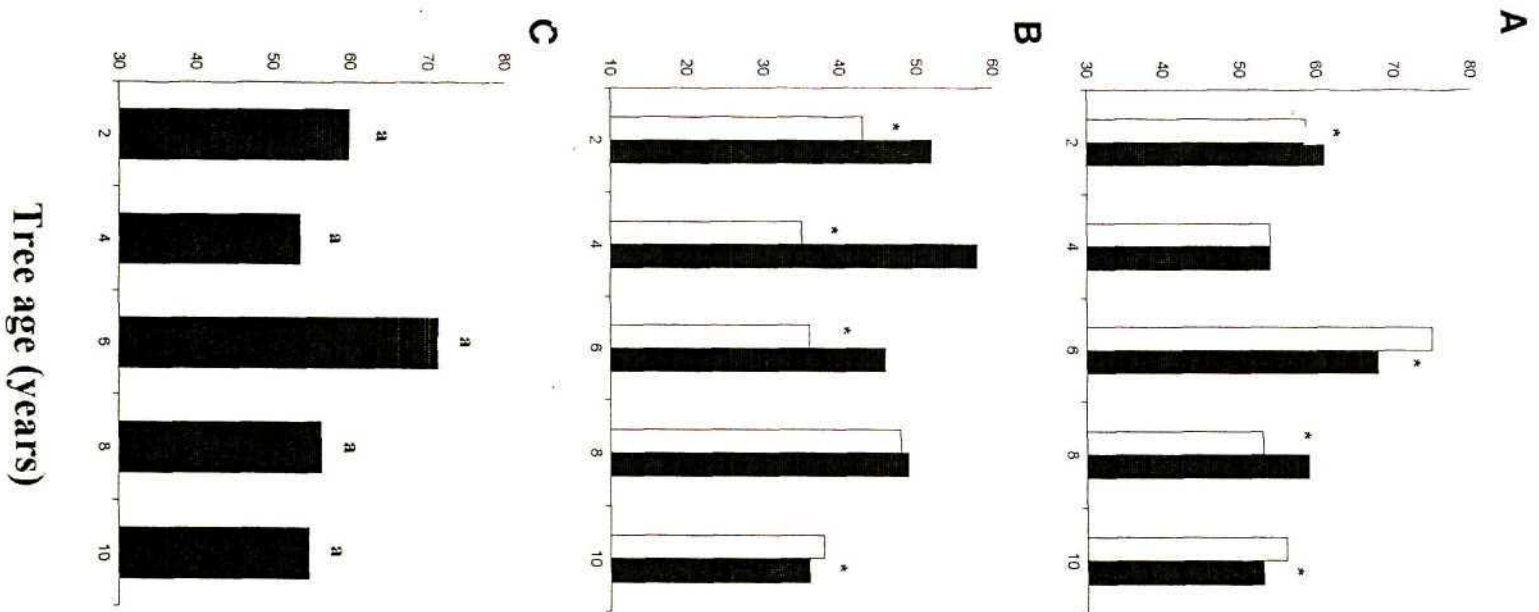
In vitro shoot production by meristems from adult and coppice material from all tree ages could be compared due to similar environmental conditions under which plant material was harvested (Appendix A, Figure 1). The conditions experienced were high temperatures (average of 28.1 °C) and high rainfall (average of 105.1 mm). When comparing percentage shooting by adult meristems with the meristems from rejuvenated coppice material, except for meristems taken from four-year-old trees, there were significant differences between the two explants sources ($p > 0.05$) (Figure 6.2 C). The results obtained are interesting as they suggest that adult material can be equally, if not more effectively, regenerated through meristem culture. This indicates that the practice of coppicing was not necessary. The results from both the adult and coppice meristems, suggest that tree age was not a limiting factor. This implies that the use of rejuvenated material such as coppice was not essential, for the black wattle. Secondly, it shows that more mature trees can be equally rejuvenated to that of younger trees.

Shoot production (%)



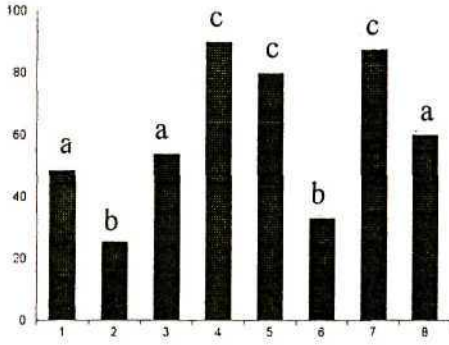
Tree age (years)

Shoot production (%)

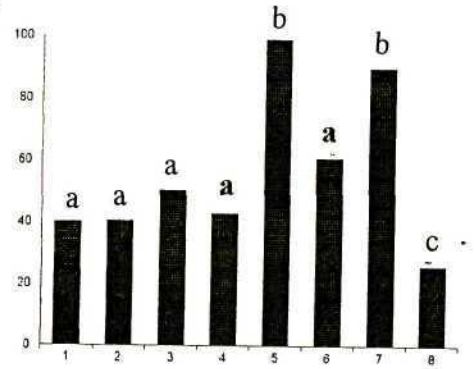


Shoot production (%)

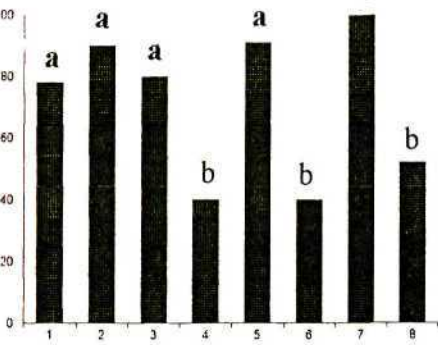
A



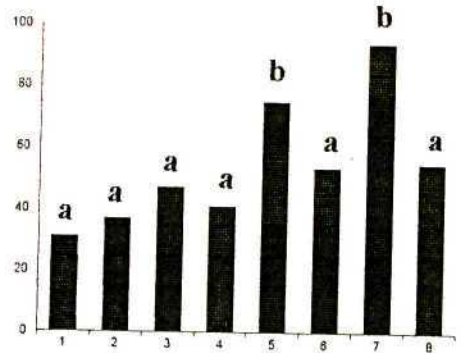
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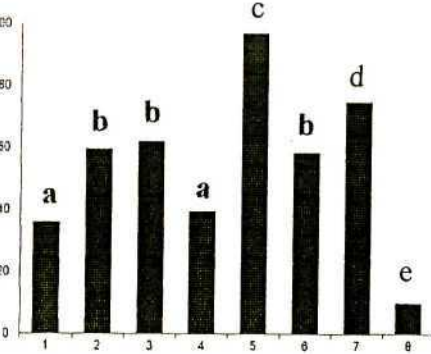
C



D



E



Treatment

6.3.2 Meristem culture of vegetative plant material from the 1998/1999 season

a) *Meristems from adult material*

In this season the use of both solid and liquid media were tested for both light and dark culture conditions. Contamination levels differed between the two types of supporting media tested (Figure 6.3). For the agar medium decontamination was successful with contamination levels being less than 10 % (except for the two-year-old material where it was 20 %) (Figure 6.3 A). With the liquid medium, contamination reached a level of 45 % (Figure 6.3 B). Thus, the agar medium was better for restricting the manifestation of contaminants (external and internal). Comparison of the two support media with respect to percentage shooting (Figure 6.4 A and B), under both light and dark conditions, showed that on average, the use of a solid support medium was more suitable for maximum shoot production and survival. The disadvantage of using liquid culture, is that it requires only one explant to get contaminated and the contamination that is produced is then rapidly distributed throughout the medium infecting the other sterile explants. On agar, only the infected explant will die, allowing sufficient time to sub-culture surrounding healthy explants. This explains the high level of contamination obtained with liquid culture (Figure 6.3 C and D) in comparison with the explants cultured on agar.

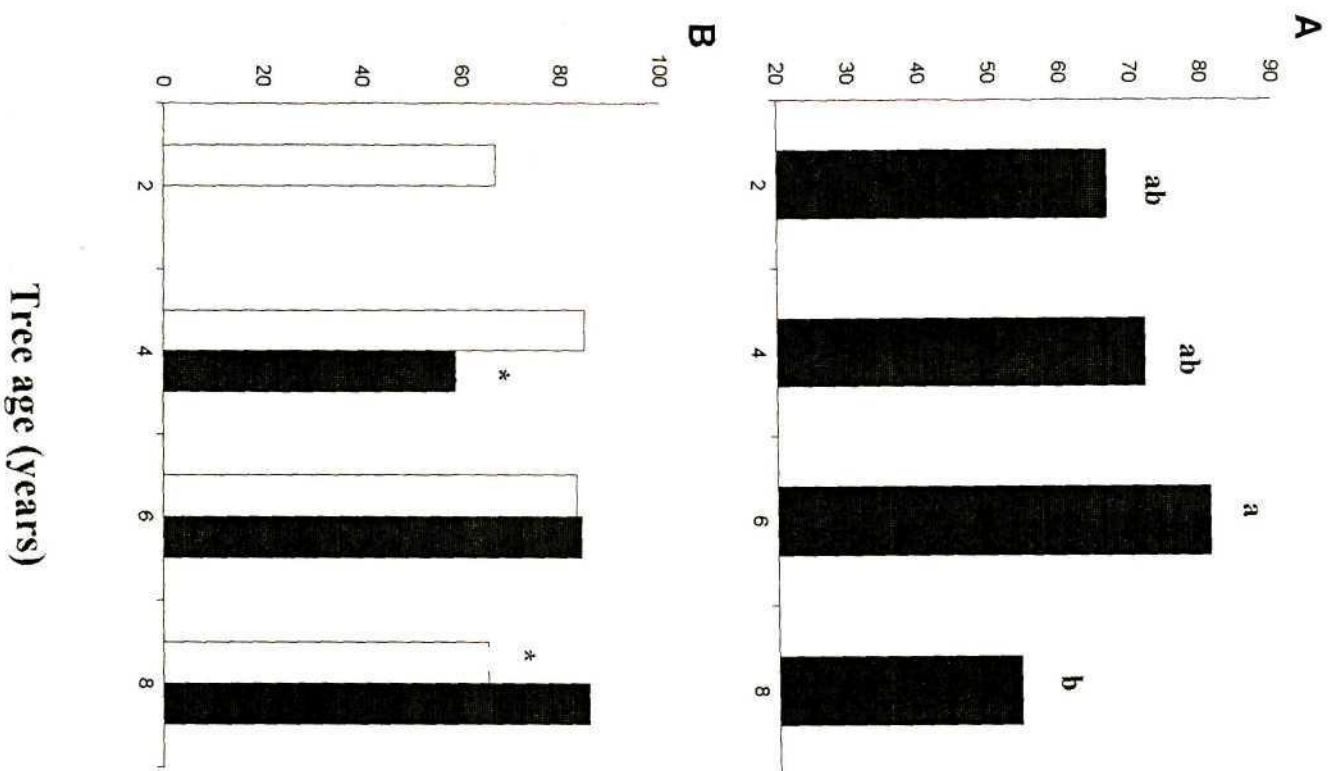
There was no significant difference between the use of light and dark culture conditions when using the agar support medium ($p < 0.05$) (Figure 6.5 A). In general, the use of dark culture conditions was superior, when working with liquid culture media (Figure 6.5 B). However, overall there was no trend indicating a difference between light and dark culture conditions for both agar and liquid supported media. Thus, looking at the total survival and shoot production of meristems on agar (Figure 6.5 C), except for the meristems taken from six-year-old trees, where shoot production was greater (71.4 %), there appeared to be no difference in percentage shoot production between the meristems taken from the various aged trees ($p < 0.05$). These results are in agreement with the results obtained in the 1997/1998 season (Figure 6.2 A). This suggests that the age of the host plant was not a limiting factor with respect to shoot production.

In order to determine the optimal medium (Table 6.1) for shoot production, it was decided to use the shoot production results from meristems cultured on the solidified media and to combine the results of the light and dark culture conditions (Figure 6.6 A, B, C, D and E). With the meristems taken from the four, eight and ten-year-old trees it was apparent that treatment 5 and 7 were best for maximum shoot production. With the meristems taken from the two and six-year-old trees, optimal shooting was achieved on a number of media. In general treatments 5 and 7 were optimal for shoot production (treatment 5 = ½ MS medium ; treatment 7 = WPM). The inclusion of L-glutamine (treatments 4 and 6) failed to enhance shooting or plantlet development when compared to the controls (treatments 3 and 5), for material taken from all trees. Only with treatment 2 did the inclusion of L-glutamine show slight improved shooting over the control (treatment 1), for meristems of all aged trees. The use of 240 medium (treatment 8) was detrimental to the growth of the explants. After a period of two months all explants producing clusters of shoots were sub-cultured onto half-strength MS medium in 100 ml culture bottles. Shoots survived for three weeks and then died.

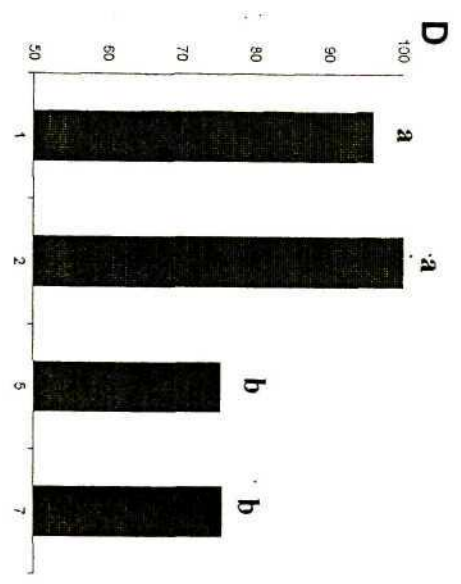
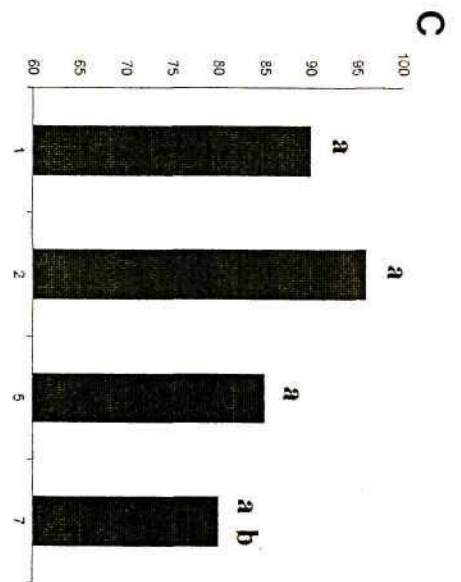
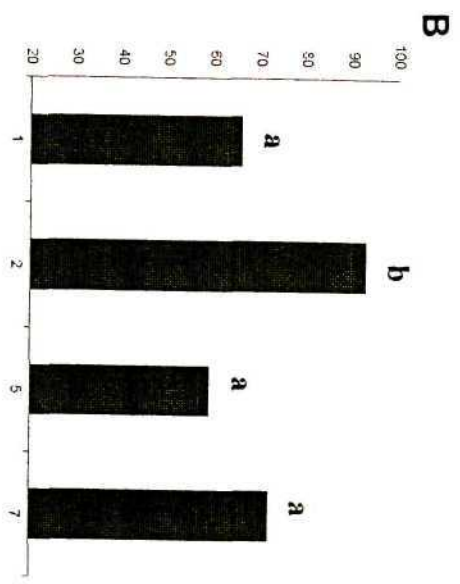
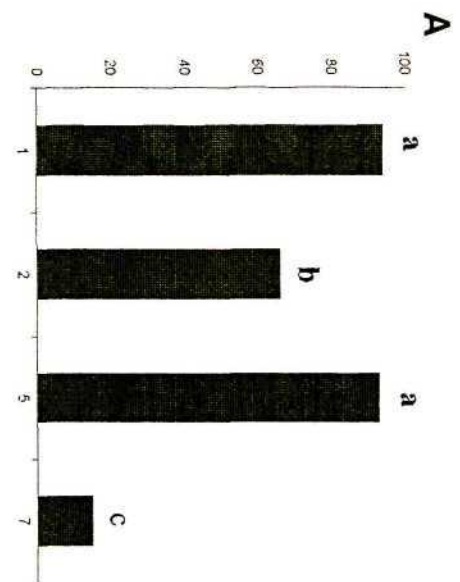
b) Meristems from coppice material

Due to the late decapitation of the ten-year-old trees, results for meristems taken from coppice produced on the ten-year-old trees were not included. In view of the results obtained with the meristems from the adult material (Section 6.3.2 a), meristems from coppice material was cultured on a solid supporting medium in the light and dark. Based on the results obtained with the meristems from the adult material, only treatments 1, 2, 5 and 7 (Table 6.1) were tested. Decontamination and shoot production was successful for meristems from coppice material produced on tree stumps for all ages tested. Contamination did not exceed 10 % and death of plant material was no more than 30 %. With reference to Figure 6.7 A, shoot production by meristems from coppice obtained from the two, four and six-year-old tree stumps, was similar ($p < 0.05$). Shoot production by coppice obtained from the two, four and eight-year-old tree stumps showed no significant differences ($p < 0.05$). In general it appears that as the trees mature, their meristematic abilities remain the same. When comparing light and dark culture conditions (Figure 6.7 B), significant differences ($p > 0.05$) were noted with meristems taken from coppice produced on the four and eight-year-old tree stumps.

Shoot production (%)



Shoot production (%)



Treatment

In general, however, it appears that it was irrelevant whether light or dark culture conditions were used. Meristems taken from coppice, produced on the two-year-old tree stumps, responded best on treatments 1 and 5 (Figure 6.8 A). Shoot production from meristems taken from coppice produced on the four and six-year-old tree stumps, was optimal on treatment 2 (Figure 6.8 B and C). Meristems excised from coppice produced on eight-year-old tree stumps, showed maximum shooting on treatments 1 and 2 (Figure 6.8 D). There was no distinct trend indicating which was the most suitable medium for maximum shoot production. Due to the failure of the adult meristems to develop further in previous experiments, shoots were sub-cultured onto MS medium supplemented with 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate and GA_3 at two different concentrations (0.1 and 1.0 mg l^{-1}) and a control supplemented only with 0.1 mg l^{-1} biotin and 0.1 mg l^{-1} calcium pantothenate. The use of gibberellins for shoot elongation has been documented (DAS and MITRA, 1990 ; JONES and VAN STADEN, 1997). Both petri dishes and culture bottles were used to establish if an increase in aeration might help plantlet development. From these results there was no trend suggesting which concentration of GA_3 was optimal and whether or not the use of petri dishes or culture bottles was more suitable for shoot elongation. The advantage of using culture bottles, however, is that they allow for increased aeration and hence plantlet development. The use of a larger explant (1.0 to 2.0 mm) and GA_3 did result in significant shoot elongation of approximately 1.0 cm (Figure 6.1 E).

c) *Comparison of shoot production by meristems from adult and coppice explants from the 1998/1999 season*

Both adult and coppice plant material was harvested under conditions of high temperatures (ranging from 25 to $26.5 \text{ }^\circ\text{C}$) and low rainfall (ranging from 59.8 to 71.45 mm) (Appendix A, Figure 1). Thus *in vitro* shoot production by meristems from adult and coppice plant material could be compared. When comparing shoot production from meristems taken from adult and the coppice material (Figure 6.9), no significant differences ($p < 0.05$) between the sources were noted by meristems from adult and coppice from all tree ages. This suggests that adult material can be used and suitably regenerated through meristem culture. This conclusion can only be confirmed once shoots have been rooted and acclimatised.

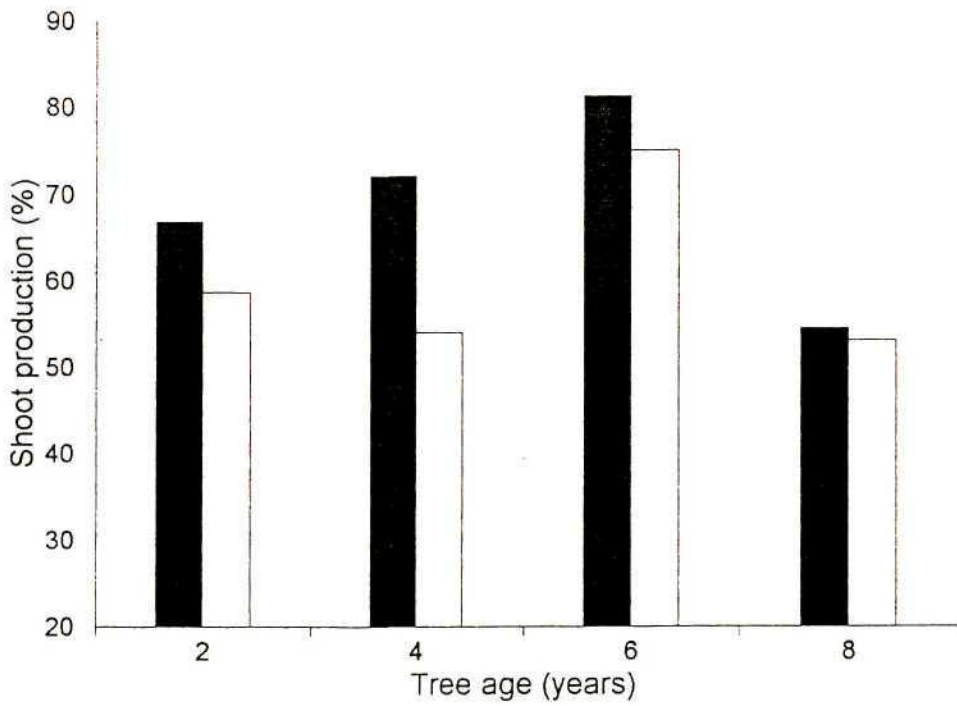
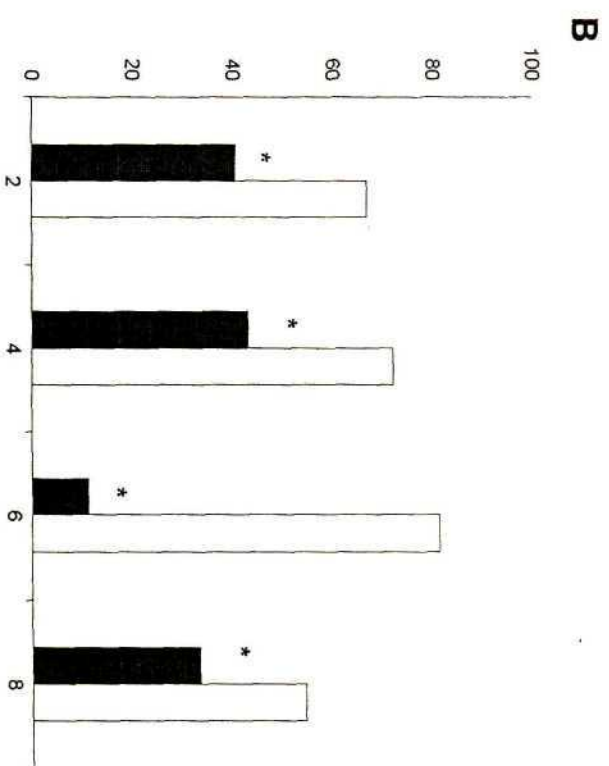
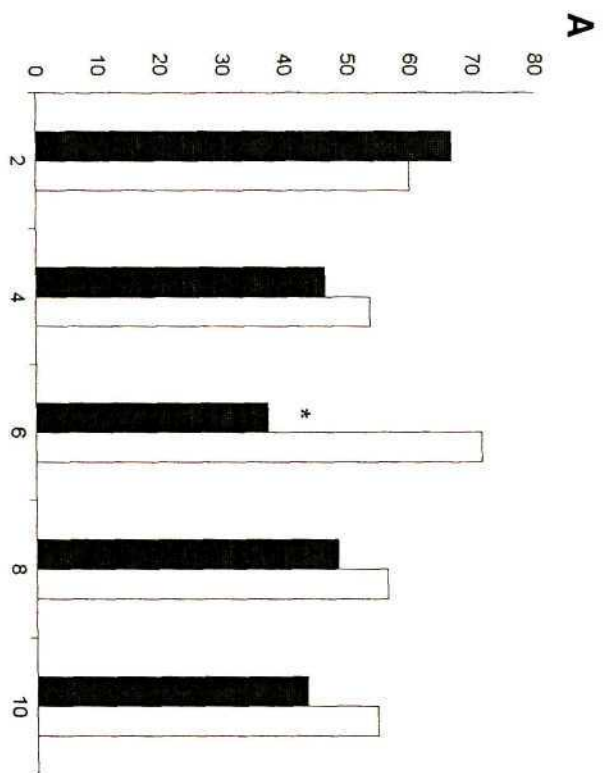


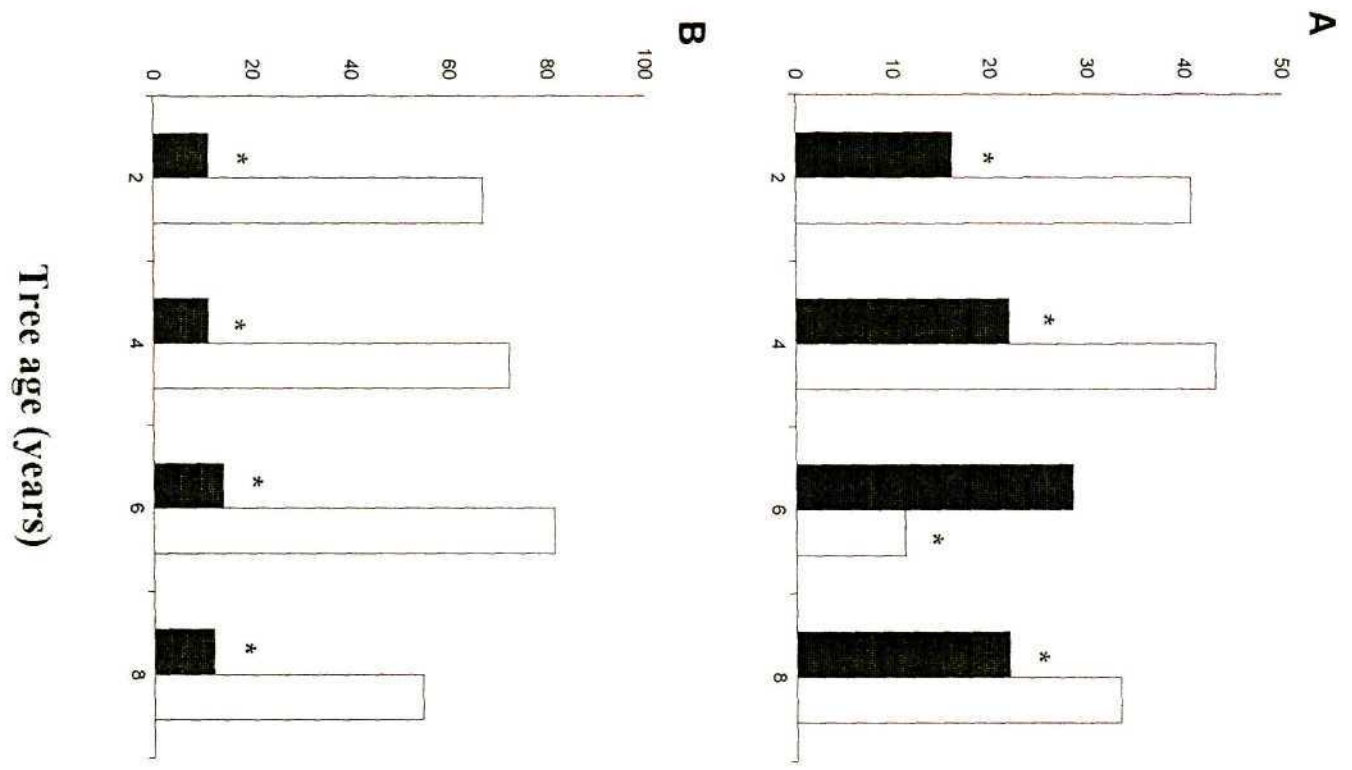
Figure 6.9 Shoot production (%) by meristems on agar-solidified medium, from adult trees (□) and from coppice (■) produced on tree stumps, of different aged *A. mearnsii* trees in the 1998/1999 season. Treatments, within a tree age, were not significantly different at the 0.05 % level.

Shoot production (%)



Tree age (years)

Shoot production (%)



Therefore eliminating the need for juvenile material in the form of coppice, to be produced. These results are in agreement with the results obtained in the previous season (Section 6.3.1 *d*, Figure 6.2 C).

6.3.3 Shoot production by meristems from various aged adult and coppice

***A. mearnsii* trees over the two seasons of experimentation (1997 to 1999)**

a) Shoot production by meristems from adult trees of different ages

Except for results obtained from the six-year-old trees, there was no significant difference ($p < 0.05$) in the results obtained over the two seasons for the adult material (Figure 6.10 A) from all five tree ages. In both seasons the conditions under which plant material was harvested, was similar with respect to temperature (ranging from 26.5 to 28.1 °C) but differed in the amount of rainfall (Appendix A, Figure 1). Rainfall in the 1997/1998 season was high (an average of 105.1 mm) and in the 1998/1999 season rainfall was low (an average of 71.45 mm). Thus indicating consistency in the results, regardless of the amount of rainfall experienced and the fact that different trees were sampled in both seasons.

b) Shoot production by meristems from coppice produced on different aged

A. mearnsii tree stumps

Meristems from coppice from all trees tested, yielded greater shoot production in the 1998/1999 season ($p > 0.05$) (Figure 6.10 B). The results could be explained due to genetic differences as different trees were used over the two seasons. Conditions under which coppice material was harvested in both seasons was similar with respect to temperature (ranging from 25 to 28.1 °C) but the amount of rainfall experienced was different (Appendix A, Figure 1). In the 1997/1998 season the rainfall was high (an average of 105.1 mm) and in the 1998/1999 season the rainfall was low (an average of 59.8 mm). The disadvantage of using coppice material, is that its production on tree stumps is influenced by environmental conditions. Thus even though shoot production *in vitro* was greater in the 1998/1999 season, the combination of high temperatures and low rainfall experienced in the 1998/1999 season limited the amount of available plant material.

6.3.4 Comparison of shoot production between meristem and nodal explants from coppice material

When comparing shoot production (Figure 6.11 A and B) on nodal and meristem explants taken from coppice material, over the two seasons examined, it was apparent that in both seasons the use of meristem explants was superior for maximum shoot production. The advantage of using meristem explants is that it eliminates the problems experienced with contamination noted when using nodal explants.

6.4 CONCLUSIONS

In vitro cloning of mature trees is generally preferred over the use of juvenile material as adult tissues have a known genetic makeup (BONGA, 1987). However, establishment of cultures *in vitro* using mature tissue is often problematic (JONES and VAN STADEN, 1997), thus the need for suitable methods of rejuvenation. The results reported in this Chapter suggest that adult trees can be successfully rejuvenated through the use of meristem culture, using both adult and coppice material. Even though only shoot production was achieved, this is an important advance in the regeneration of adult plant material. These conclusions will have to be confirmed once shoots have been rooted and acclimatised. It was apparent that the culture of meristems from the coppice material was not necessary as shoot production from the meristems from adult material was equal, if not better than that obtained from the coppice material. This is important as it eliminates the need to use rejuvenated plant material such as coppice. It was also evident that over the two seasons, the results obtained from meristems taken from the adult material remained consistent. Whereas the meristems from coppice material varied over the two seasons. The disadvantage of using coppice plant material is that the time of felling of the trees for coppice production, is highly dependant on rainfall, which in itself is unpredictable. The amount of coppice produced on the decapitated tree stumps was also limited due low rainfall experienced in the 1998/1999 season. Thus emphasizing the advantage of using adult material. Mature trees of superior quality can thus be selected, rejuvenated and regenerated *in vitro* using meristems from adult material. In both seasons (1997 to 1999) the age of the parent tree, from which plant material was obtained, did not appear to have a significant effect on the degree of shoot production. This too was important

as it implies that plant material can be taken from mature trees and can be successfully rejuvenated and regenerated *in vitro*, equal to that of plant material taken from younger trees. Thus the age of the plant material was not a limiting factor. Multiple shoot production was possible from meristems taken from both adult and coppice plant material from all tree ages tested. However, due to the small size of the material under examination, it was impossible to analyze the number of shoot primordia produced per explant. Thus, all results were expressed as the number of explants producing shoot buds. The use of WPM and half-strength MS medium for the culture of meristems from both adult and coppice material, proved to be the best for shoot production. In the 1998/1999 season, no significant difference ($p < 0.05$) was evident between the use of light and dark culture conditions. And it was apparent that the use of a solidified agar medium was superior for meristem culture. The problem encountered with using meristem culture was the difficulty in promoting shoot elongation. This problem is not unusual when using meristem culture as a technique (DODDS and ROBERTS, 1985 ; BARAKAT and EL-LAKANY, 1992). It was for this reason that the use of gibberellic acid was tested, together with increasing the size of the excised explant from 0.5 to 1.5 mm. BARAKAT and EL-LAKANY (1992) used *Acacia mangium* explants ranging from 2 to 3 cm in length. The use of a larger explant together with GA₃ helped increase shoot elongation (which was approximately 1.0 cm) and shoot development. *Pinus pinaster* meristems were successfully elongated using low concentrations of BA and high supplementation of sucrose in the medium (HACKETT, 1985). However, optimal shoot elongation and further development still needs to be maximised. Due to the small size of the excised explant and the requirements for the morphogenic development, in future a softer support medium such as Gelrite should be used as this would possibly increase mobilization of nutrients (BORNMAN and VOGELMANN, 1984 ; WETZSTEIN, CHOONGSIK and SOMMER, 1994). The advantage of using meristem culture over that of organogenesis through nodal culture, is that contamination was almost eliminated and survival rates were far greater.

The results obtained from the use of meristem explants, noted in this Chapter, are relevant to the forestry industry in the need to overcome maturation effects and promote the rejuvenation of adult material. The observations made in this study indicate that adult material can be sufficiently regenerated through the use of meristems taken from apical tips of adult trees. This

however, will have to be confirmed once plantlets have regenerated and successfully introduced into the field. The use of meristems from rejuvenated material such as coppice, did not show any advantage in generating multiple shoots, over that of meristems from mature plant material. These results held for both of the seasons tested. The results obtained are novel for this species and pertinent to the efficient propagation of woody forest trees. However, the research is still in its developmental stages as shoot elongation, rooting and subsequent acclimatisation still needs to be optimised. The progress obtained thus far offers a large amount of scope for further work in this area. Meristem culture thus eliminates the effect of maturation, reduces contamination thus increasing *in vitro* success by using the apical tip, which is considered pathogen-free. In future years this technique will hopefully allow for simple regeneration of adult material and the production of trees less susceptible to disease. Once all areas of the technique have been optimised and the system is operating, meristem culture will allow for easy amplification of tissue and could then be incorporated into a future clonal programme of the black wattle.

CHAPTER 7

TANNIN PRODUCTION AS A POSSIBLE PHASE TRANSITIONAL MARKER

7.1 INTRODUCTION

In the eighteenth century the vegetable tannin industry was smaller than the hide industry, however, it produced the largest expenditure on raw materials at the tanneries. These materials were priced on the tannin content. The problem with tannins is that they are not a defined chemical entity, which can be precisely evaluated. The first method of tannin analysis to be acknowledged, was devised by LÖWENTHAL in 1877, involving volumetric analysis. This method depended on the oxidation of tannins by potassium permanganate under slightly acidic conditions. The detannisation or removal of tannins from the system proved to be the weak point in this method. Since removal of tannins with metals and gelatine was unsuccessful, it was recommended that skin-collagen be used. A non-powdery hide powder was required that could be washed free of all solubles and allowed for no interference with water solubles. It was for this reason that the hide powder produced was slightly chromed. In 1886 SIMAND and WEISS developed such a method using hide powder. By simply macerating the hide powder with the tannin solution and filtering the mixture which had been detanned and then drying it, the difference in residues of the initial solution to that of the non-tans allowed for the determination of tannins. This method forms the basis of tannin analysis used today. In 1897 PROCTOR made an improvement where he introduced the Filter Bell method. In this method the hide powder was packed uniformly into a bell ended tube which was submerged in the analytical solution and the solution was then syphoned at a specific rate through the bell (WAITE, 1991).

For a number of years the wattle growers have required a suitable method to grade bark using chemical analysis. This, however, is tedious and results for percentage tannins are not absolute values. The percentage tannin in a bark sample is based on the aqueous extraction of a defined

weight of bark in a Proctor extractor at controlled temperatures. Fifty milliliters of the extracted solution is evaporated to dryness ; 100 ml is shaken with hide powder to remove the tannins and 50 ml of the non-tan solution is evaporated to dryness. The remainder of the solution is filtered and 50 ml is evaporated. The residues of the total solids, total solubles and non-tans, are air-dried in ovens, desiccated and weighed. From the difference between the total solubles and non-tan residues, the tannin content can be determined (GORDON-GRAY,1954).

A number of precautions need to be taken when analyzing tannins in order to reduce variation in samples and increase the consistency of the technique (GORDON-GRAY,1954). Tannin analysis tests need to be specifically defined if they are to be of any scientific value. Three such specifications are required to be defined for successful tannin analysis, namely, the method of filtration, the type and quality of hide powder used and the method of detannisation (WAITE,1991). Standardizing of equipment, macerating the bark samples prior to testing and obtaining the absolute moisture content of the bark samples all help increase the reproducibility of this technique (GORDON-GRAY,1954).

The hide powder analysis of tannin content of materials requires precision and demands close control of conditions of analysis. One such condition is the quality and type of hide powder used, variations can lead to erratic results. Investigations into an alternative standard were conducted. The best reagent identified was microcrystalline diethylaminoethyl cellulose (DE32) in the sulphate form. This reagent may be regenerated numerous times without losing its analytical precision. Experiments were conducted using Mimosa extracts and the non-tans were analyzed. Analysis was conducted using DE32 unused, DE32 regenerated ten times, and standard hide powders of good quality, and there was no difference in the results. The advantage of DE32 is that it is readily available in a synthetic form. It can be used in the dry form eliminating a dilution factor and allows for large quantities to be made up at a time thus reducing time. It can also be regenerated up to 20 times without loss of analytical precision (GARBUIT and NOBLE,1990).

The search for a biochemical marker or cytological marker which is an early indicator of the transition to the mature phase and an intrinsic indicator of differentiation in the cells of juvenile and mature cells, is an important area of study (HACKETT, 1985). Morphological and chemical markers have been used to identify the most juvenile cells, the best tissue and best time to collect it (BONGA and VON ADERKAS, 1992).

Freshly excised tissues of woody plants, especially angiosperms, often secrete brown pigments consisting of oxidised polyphenols and tannins into the medium, in response to wounding (THORPE, HARRY and KUMAR, 1991). One of the most important characteristics of the black wattle is its bark, as this is one of the richest known sources of tannins (ROUX, KEMP and WINGFIELD, 1995). Phenolics inhibit shoot growth and can kill plant material (THORPE, HARRY and KUMAR, 1991). Phenolic production is also problematic in the establishment of cultures *in vitro* (JONES and VAN STADEN, 1997). Explants from more mature tissues have increased levels of phenolics (THORPE, HARRY and KUMAR, 1991), thus tannin production may be an indicator of the transition from juvenility to maturity.

7.2 MATERIALS AND METHODS

7.2.1 Plant material

Bark samples were collected from trees from five age groups, namely, two, four, six, eight and ten years of age. In each age group there were ten samples.

7.2.2 Tannin analysis

Tannin analysis was conducted at the Institute for Commercial Forestry Research (ICFR). For the preparation all bark samples were air dried until the bark was brittle. The bark was cut into matchstick size pieces and milled through a 0.5 mm mesh and the sample was bottled. Tannin analysis involved weighing 10.5 g of the air dried samples in duplicate. These samples were then dried for two hours in a vacuum oven, cooled and the dry weight was determined. Samples were refluxed for two hours using 400 ml de-ionized water. Extracts were transferred into a 1 l volumetric flask and made up to volume. Samples were placed on ice (approximately 10 ° C) and cooled to 18 ° C and then made up to one liter (solution A). The total solids were

determined from the residue weight of solution A. The total solubles were determined from the residue of filtered solution A. The non-tannins were determined after the tannins were removed from solution A by extraction with diethylaminoethyl cellulose (DE32) and kaolin followed by filtration. The residue weights were determined by evaporating 50 ml of solution to dryness in pre-weighed basins on a boiling water bath, followed by drying in a vacuum oven for two hours, cooling and re-weighing.

7.2.3 Statistical analysis of results

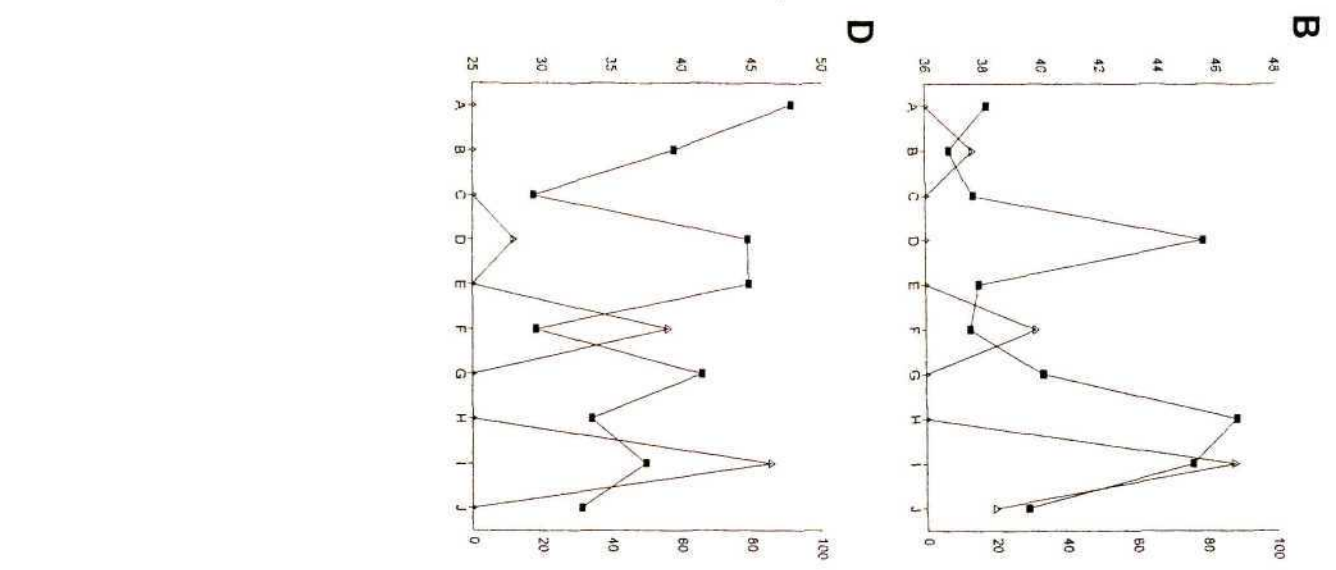
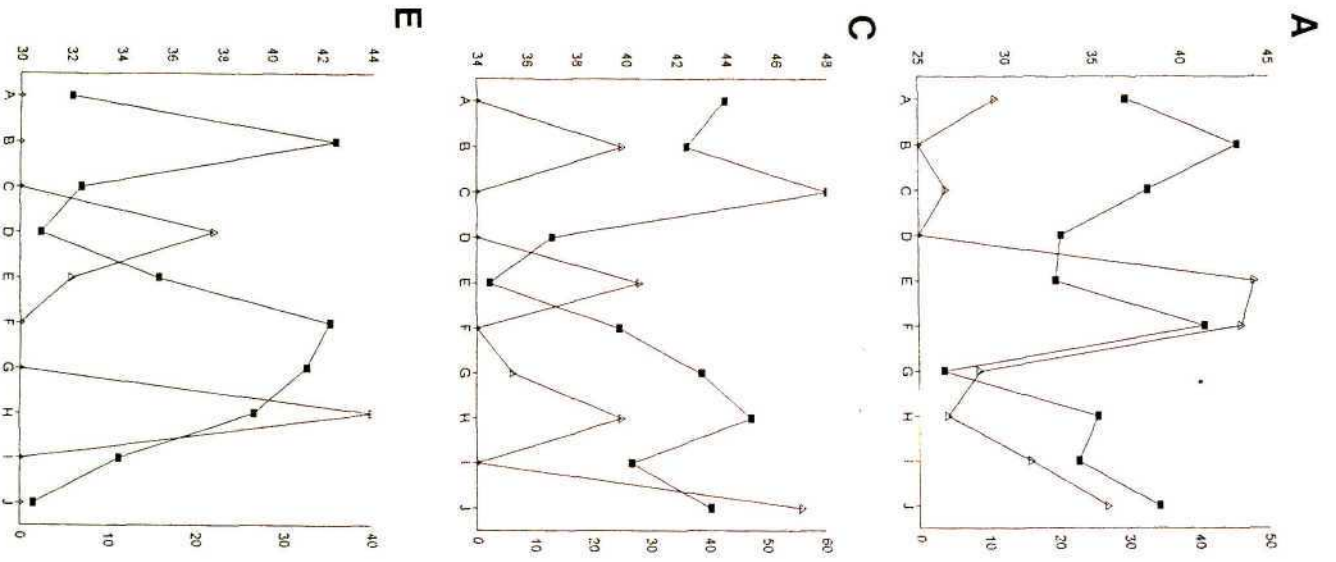
A correlation co-efficient was calculated for the percentage tannin production versus percentage shoot production. Significance was tested at the 95 % level.

7.3 RESULTS AND DISCUSSION

7.3.1 Tannin analysis

Ten samples from each tree age group were analyzed for tannin content. Based on the literature, phenolics are known to inhibit regeneration and meristematic growth (THORPE, HARRY and KUMAR, 1991), for this reason it was expected that as tannin levels increased so the percentage shoot production would decrease. With reference to the two, eight and ten-year-old bark samples (Figure 7.1 A, D and E), this trend appeared to exist, however, statistically there was no correlation ($r=0.087$; -0.253 and 0.018 , respectively when $p= 0.05$). However, results from the bark samples taken from the four and six-year-old age groups (Figure 7.1 B and C) were inconsistent ($r=0.261$ and 0.00038 , respectively).

In each age group there were marginal differences in the percentage tannins extracted from the ten bark samples. Thus illustrating consistency in the tree samples tested. When looking at the entire group of bark samples in each age group (Figure 7.2), there appears to be a slight increase, in percentage tannins extracted, as the tree age increases from two to six years of age. Thereafter percentage tannins decreases slightly with tree age. There was no correlation ($r=0.643$ when $p= 0.05$) between percentage tannins extracted and shoot production as the tree age increased.



Tree replicates

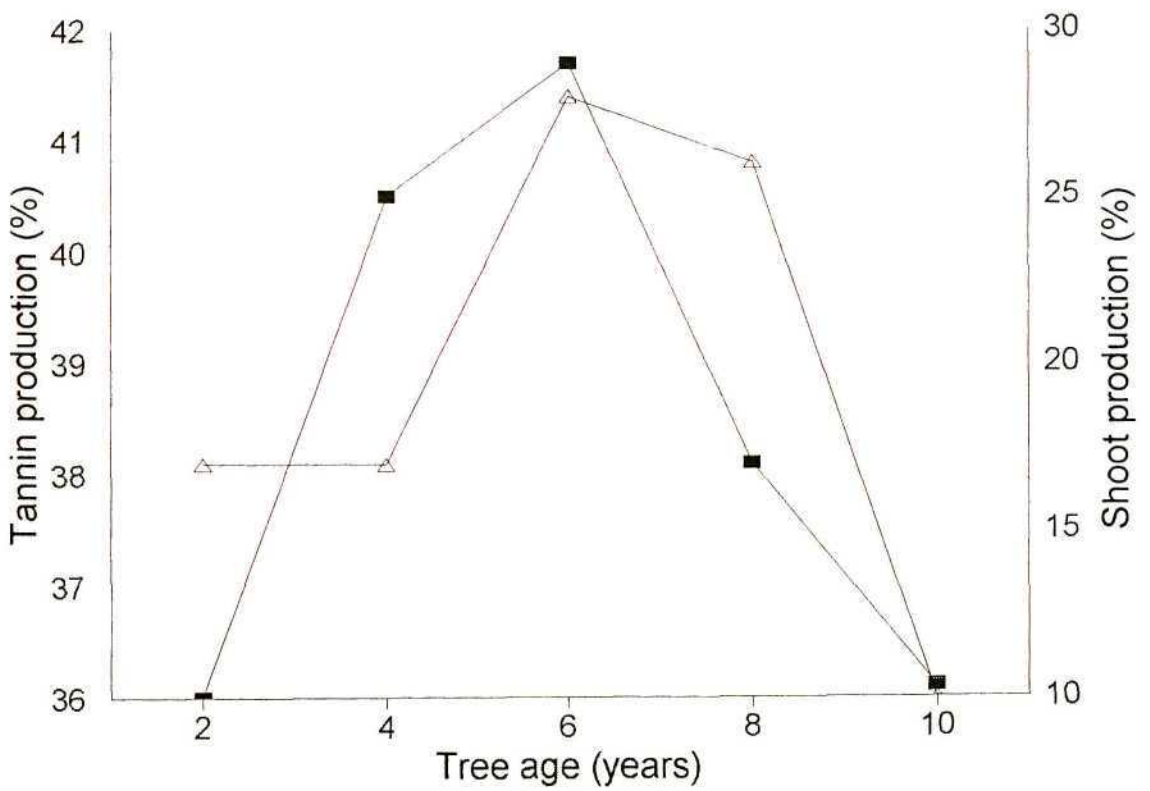


Figure 7.2 Percentage tannin extracted (-■-) from bark samples versus shoot production (-Δ-) from coppice produced on different aged *A. mearnsii* trees. A correlation co-efficient was calculated at the 95% level.

However, there was no indication that tannin production had any effect on shoot production *in vitro* (THORPE, HARRY and KUMAR, 1991).

7.4 CONCLUSION

The results achieved from this study are inconsistent and thus it was difficult to extract many conclusions. The percentage tannin extracted from individual bark samples within each age group differed little to the mean, thus illustrating consistency in the samples chosen. However, results from the percentage shoot production, from nodal coppice material produced on the tree stumps in each age group, showed no correlation to the percentage tannins present. Thus it could not be deduced whether or not tannin production altered plantlet regeneration and if it was an indicator of a transition from the juvenile to the adult phase. A more in depth study would have to be conducted, where physiological and morphological analysis were undertaken.

CHAPTER 8

CONCLUSIONS, FUTURE PROSPECTS AND RELEVANCE

8.1 CONCLUSIONS

During the past few years, there has been a significant increase in the use of tissue culture in clonal forestry as an alternative to conventional vegetative practices. Extensive *in vitro* research and success has been achieved for a number of forest trees, including the *Pinus*, *Eucalyptus* and *Acacia* species. However, little attention has been given to the black wattle (*Acacia mearnsii*) in particular. The aims of this study were to investigate the micropropagation of *A. mearnsii*. The work conducted in this study clearly showed that *in vitro* plantlet regeneration of the black wattle can be achieved and the rejuvenation of adult material through the use of coppice and apical tips, was successful, thus satisfying the initial objectives of this study. Considerable advances have been made in this study since the first reported work conducted on *A. mearnsii in vitro* (GONG, AL-KHAYRI and HUANG, 1991 ; HUANG, AL-KHAYRI and GBUR, 1994). In this report *A. mearnsii* was regenerated *in vitro* using shoot tips from 30-day-old *in vitro* plants grown from seed. Since this report, the introduction of *ex vitro* material into culture and the regeneration thereof has been achieved (BECK, DUNLOP and VAN STADEN, 1998 a). The *in vitro* rejuvenation of adult material through the use of coppice material from five various age groups has been recorded (BECK, DUNLOP and VAN STADEN, 1998 b). Important advances have been made using meristem culture from both adult and juvenile material, in rejuvenating adult plant material, thus making the results of this study both novel and beneficial for the future of the wattle industry. A preliminary study was conducted on the analysis of tannins from bark extracts from adult trees. Tannins are phenolics and are known to inhibit *in vitro* development of plant material (THORPE, HARRY and KUMAR, 1991). Tannin production is also an indication of the transition from a juvenile state to maturity (HACKETT, 1985). Thus the study was aimed at determining if tannin production influenced *in vitro* performance and if possible could be an indicator of this transition from juvenility to maturity. Results were inconclusive, however, with

a more in depth physiological study, results achieved could be valuable. A number of problems were encountered and still need to be resolved for these results to be applied on a commercial scale in clonal forestry. Successful *in vitro* rooting and acclimatisation of rejuvenated shoots from coppice material still needs to be achieved. Elongation and plantlet regeneration of *in vitro* regenerated meristems still needs to be optimised, in order that one can conclude the advantages of meristem culture in the rejuvenation of adult material. Furthermore, the control of endogenous contaminants through the use of antibiotics needs to be addressed. However, due to a limited amount of time these could not be completed.

Tissue culture techniques have been incorporated into clonal programmes for both the *Eucalyptus* and *Pinus* species and thus there is no reason why tissue culture should not be applied to the propagation of the black wattle. The black wattle is fast becoming one of the most important trees in forestry in South Africa. For this reason it is important to employ biotechnology which would enable transformation, and genetic modification, as well as storage of superior germplasm thus servicing the needs of the industry. Thus the results from this study have shown that the black wattle too, can be cultured *in vitro*. These results, once optimised, could be incorporated into a breeding programme for the wattle industry.

8.2 FUTURE PROSPECTS

Future research lies in optimising the protocol for the tissue culture of the black wattle so that this technology can be conducted on a commercial scale and ultimately be incorporated into a clonal programme for *A. mearnsii*. However, various problems encountered in this study need to be overcome in order to allow for successful *in vitro* regeneration of adult plant material, either via meristem culture or through the use of coppice material. The incorporation of antibiotics into a sterilization programme to control infection is a possibility that requires attention. The use of gibberellins for the elongation of shoots generated from meristems and the use of various carbohydrates and solidifying agents, to increase availability of nutrients are areas of future research. The results achieved thus far fulfill the aims of this study and have opened up a wide avenue for future development of the black wattle. *In vitro* research on the wattle is far behind that of other woody species and especially other forest trees, however, the achievements obtained from this study will, in time, put the black wattle on a par with research being conducted on

various *Pinus* and *Eucalyptus* species. The limited documented work conducted *in vitro*, on the *Acacia* species, allows for a wide scope for future research.

8.3 RELEVANCE AND IMPORTANCE OF THIS STUDY

Tissue culture compared to conventional vegetative practices, offers a number of advantages for the propagation of forest trees. It allows for the initial bulking up of plant material in a relatively short period of time, clonal material in particular. Plant material can be cultured and worked all year round due to the manipulation of culture conditions, thus plant material is not susceptible to seasonal changes. It can aid in rejuvenation of adult tissue, thus eliminating the effect of maturation which is a major problem. Tissue culture of seedling material can be advantageous in breeding programmes and eliminates seed production. Commercial production of genotypes can be produced in one to two years after selection opposed to four to ten years using traditional methods. Breeding will always be important for new genotypes which can then be multiplied asexually. Multiplication of coppice allows for the propagation of superior adult trees. Rejuvenation of mature tissue has been overcome through meristem culture and somatic embryogenesis (FURZE and CRESSWELL, 1985). The use of tissue culture for the production of more uniform plants is important in clonal forestry which can result in the economic processing of timber. The use of tissue culture on a commercial basis is always limited due to financial constraints. In Italy the axillary bud technique is being used to propagate *Eucalyptus* species. Mondi in South Africa is using tissue culture to establish cold-tolerant *Eucalyptus* clones *in vitro* and over 80 % rooting has been achieved. Advanced Technologies Cambridge (ACT) in the United Kingdom have incorporated tissue culture for Eucalypt biotechnology. Various companies have started using genetic biotechnology to identify clones and various markers have been identified for various traits eg. fibre, wood density, disease, thus allowing for early identification for selection. Similar genetic improvements have been achieved through transformation technology to include genes of importance. Genetic manipulation offers an opportunity to improve the quality of selected material. Tissue culture allows for the rapid multiplication of various traits, however, the effectiveness of such techniques is dependant on field performance (JONES and VAN STADEN, 1997).

Tissue culture also allows for the genetic manipulation and transformation of plant material, resulting in the production of trees more desirable to the industry e.g. trees with superior timber or bark qualities or ultimately sterile trees. Another important tool associated with tissue culture is cryopreservation. This enables one to store plant material which has been genetically transformed or crossed, for an unlimited period of time. Plant material can then be tested in the field and the superior clones can be selected and then removed from cryopreservation and bulked up. Tissue culture is, however, initially very costly. Only once the technique has been developed for the specific plant material and the method is efficient, can it be cost efficient. Even though the establishment of a suitable tissue culture method for the black wattle is still in the developmental stages, many successful steps have been made and it will be a matter of time before this technique will form an integral part of a future breeding and clonal programme. Tissue culture is an extremely useful tool with many advantages and should not be dismissed due to expenses. Tissue culture is becoming an integral part of breeding programmes (FURZE and CRESSWELL, 1985) and hopefully results obtained from this study will be incorporated into the forestry industry.

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APPENDIX A

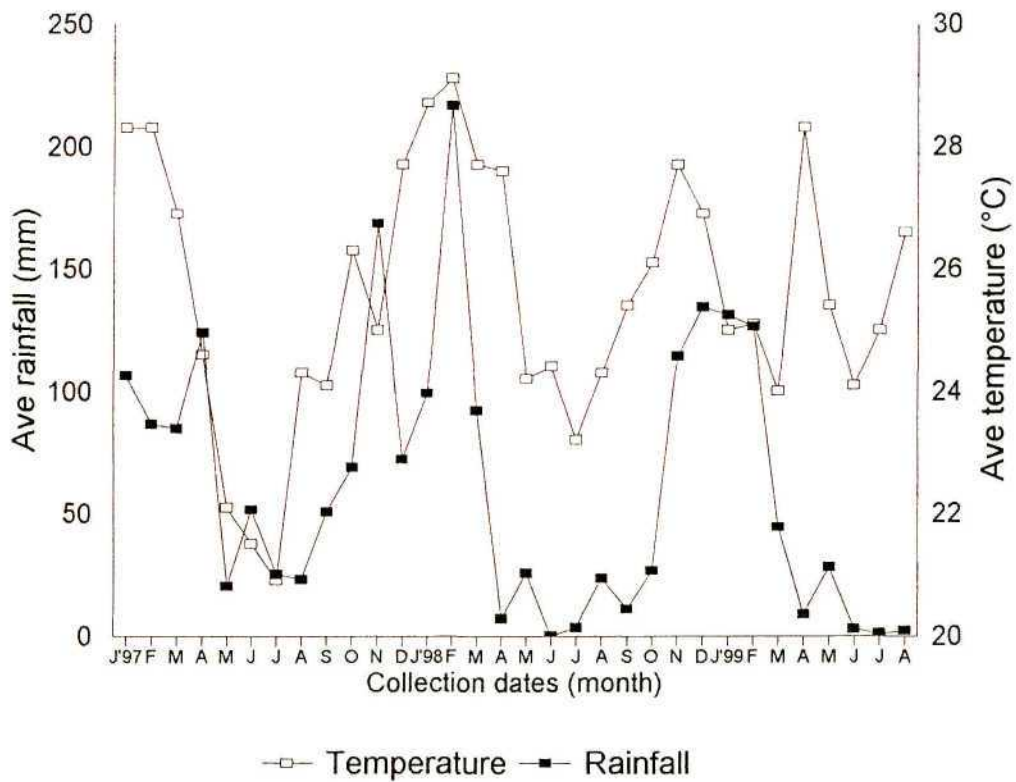


Figure 1 Monthly rainfall and temperature data for the period 01/01/1997 to 31/08/1999, measured at the Darvill Sewage Purification Works. Data was provided by the Weather Bureau, Durban.

APPENDIX B

Table 1. Values of the correlation co-efficient for significance by a two-tail test

Degrees of Freedom	0.05
1	0.9969
2	0.9500
3	0.8783
4	0.8114
5	0.7545
6	0.7067
7	0.6664
8	0.6319
9	0.6021
10	0.5760