SHOOT APEX CULTURE OF *ACACIA MEARNSII* (DE WILD)

BY

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ABSTRACT

Research into the micropropagation of black wattle in South Africa is important for two reasons. Firstly micropropagation technology allows breeders to select and propagate mature tissue, which in turn allows them to better capture selected traits. Secondly, tissue culture may control the highly invasive nature of black wattle. If triploid black wattle can be developed, foresters will then have to rely on clonal propagation to supply material for their growing operations. This research was part of the Institute for Commercial Forestry's *Acacia mearnsii* vegetative propagation programme. The main focus of this research was to overcome various problems associated with direct organogenesis of *ex vitro* material. The shoot apex region was used as the explant in all studies because this region is thought to harbour relatively few internal microbial contaminants and is of sufficient size to withstand stresses associated with micropropagation.

The initial research was focussed on the screening of sterilants, searching for a viable alternative to mercuric chloride. Surface sterilisation is integral to any micropropagation technique. This process should do the least amount of plant damage, whilst reducing microbial contamination to an acceptable level. Explants were cultured on Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} BA and monitored for signs of contamination and shooting. Household bleach proved an excellent alternative to mercuric chloride because it did significantly less damage to the explants than mercuric chloride and is handled easily. There was no significant effect of sterilant exposure time on explant decontamination levels, whilst the shortest exposure time resulted in significantly higher levels of shoot development than the other two times tested. The results of this initial research was developed into a protocol and utilised in subsequent investigations.

Due to a considerable variation in the success of the developed surface sterilisation protocol according to different times of the year, a further investigation into the effects of season and mother plant material on shoot apex culture of *Acacia mearnsii* was undertaken. The success of any tissue culture technique depends on a large array of *ex vitro* and *in vitro* variables. The objective of this research was to determine the

effect of two *ex vitro* variables, season and mother plant, on shoot apex culture of *Acacia mearnsii*. Explants from individual mother plants were cultured on MS medium supplemented with 2.0 mg L^{-1} BA during four separate seasons and monitored for signs of contamination and shooting. Spring was found to be the best harvesting season because spring explants showed significantly higher decontaminated explant levels and shooting levels than explants harvested in the other three seasons. The effect of mother plant selection on the performance of *Acacia mearnsii* explants during shoot apex culture was also found to be significant, especially with regard to shooting levels.

Finally factors influencing shoot elongation of *A. mearnsii* during shoot apex culture were investigated. In the past, induction of shoot elongation during micropropagation of *A. mearnsii* was attained through the addition of plant growth regulators and other supplements to the basal culture medium. However, some micropropagation methods in other species have utilised red light as a means of promoting shoot elongation. The objective of this study was to test the effects of an alternative basal medium, red light and differing concentrations of chemical additions to the culture medium on shoot elongation of *Acacia mearnsii* during shoot apex culture. Four independent experiments were undertaken comparing: shoot elongation on Woody Plant Medium (WPM) to the MS basal medium control; shoot elongation under a red cellophane box compared to control culture light conditions; shoot elongation on media supplemented with various concentrations of GA_3 to the un-supplemented control and shoot elongation on media supplemented with combinations of BA and IBA compared to a control. Although no significant effects were observed, many trends were noted. The results indicated that there was no advantage to using WPM instead of MS medium when attempting to elongate shoots, rejuvenated through shoot apex culture of *A. mearnsii*, whilst the effect of GA_3 showed a negative trend. The effects of red light and some BA and IBA combinations showed positive trends on the elongation of initiated shoots.

This research successfully addressed some of the problems associated with micropropagation of *A. mearnsii*. Shoot apex culture shows promise and further research into this technique should be considered. A viable surface sterilant alternative to mercuric chloride was successfully identified. This alternative is not only

safer to use but shows a large reduction in phytotoxic effects. The effects of season and mother plant on shoot apex culture was successfully investigated, resulting in a better understanding of mother plant influences on tissue culture as well as the identification of an optimum season for explant selection. Finally two possible shoot elongation promoters were identified for further research and a more affordable alternative to red light sources and screens was identified.

DECLARATION

The experimental work described in this thesis was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Prof. Mark Laing and the co-supervision of Dr. Annabel Fossey and Dr. Sascha Paye.

These studies represent original work by the author and have not otherwise been submitted in any other form to another university. Where use has been made of the work of others, it is duly acknowledged in the text.

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NOVEMBER 2007

I hereby certify that this statement is correct.

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FOREWORD

All research presented in this thesis was conducted at the Institute for Commercial Forestry Research on the premises of the University of KwaZulu-Natal, Pietermaritzburg, South Africa. The work presented is the culmination of five years of research which started as an honours project and developed into a Masters thesis. The majority of the laboratory work was conducted in the first three years.

As part of the Institute for Commercial Forestry's *Acacia mearnsii* vegetative propagation programme, the main focus of this research was to overcome various problems associated with direct organogenesis of *ex vitro* material, using small virus-reducing explants in *Acacia mearnsii*. The shoot apex region was used as the explant in all studies because this region is thought to carry a low level of internal microbial contaminants, and is of sufficient size to withstand stresses associated with micropropagation.

Ultimately, it is hoped that a viable protocol for shoot apex culture of mature black wattle can be achieved and in order for this goal to be attained various problems must be eliminated. One such problem is the resistance shoots show to elongation once induced. Surface sterilisation of *ex vitro* grown black wattle explants, whilst not identified as a problem, has in the past made use of mercuric chloride, a powerful toxin. The focus of this study was therefore:

1. To investigate the effects of different sterilisation agents and exposure times on cultured *Acacia mearnsii* shoot apex contamination levels.

2. To test the effects of harvest season and individual mother plant on shoot initiation during shoot apex culture of *Acacia mearnsii*.

3. To investigate the effects of physical and hormonal factors on shoot elongation of *Acacia mearnsii* during shoot apex culture.

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CONTENTS

Abstract.....i

Declaration.....iv

Foreword.....v

Acknowledgements.....vi

Contents.....vii

Chapter 1: Literature review.....1

1.1 Introduction.....1

1.2 Forestry in South Africa.....1

1.3 Black wattle industry in South Africa.....3

1.4 Black wattle tree improvement……..…5

1.5 Vegetative propagation……12

1.6 Micropropagation……..…13

1.7 Applications of micropropagation in black wattle……20

References……..…...23

Chapter 2: Screening of topical sterilants for shoot apex culture of *Acacia mearnsii*.....28

Abstract.....28

2.1 Introduction.....28

2.2 Materials and methods.....30

2.3 Results.....34
2.4 Discussion.....38
References.....40

Chapter 3: Effect of harvesting season and mother plant on on shoot apex culture of *Acacia mearnsii*.....42

Abstract.....42
3.1 Introduction.....42
3.2 Materials and methods.....43
3.3 Results.....46
3.4 Discussion.....53
References.....57

Chapter 4: Effect of red light and an alternative basal medium shoot elongation of *Acacia mearnsii* during shoot apex culture....58

Abstract.....58
4.1 Introduction.....58
4.2 Materials and methods.....60
4.3 Results.....66
4.4 Discussion.....69
References.....70

Chapter 5: Effects of plant hormones on shoot elongation of *Acacia mearnsii* during shoot apex culture……72

Abstract.....72
5.1 Introduction.....72
5.2 Materials and methods.....73
5.3 Results.....78
5.4 Discussion.....81
References.....82

Chapter 6: Thesis overview……84

6.1 Introduction.....84
6.2 Screening of sterilisation agents and exposure time.....85
6.3 Seasonal and mother plant effects.....86
6.4 Hormonal and physical effects on shoot elongation.....87
6.5 Overall conclusion…….....89
References.....89

Appendix 1.....91

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

In the 21st century forestry has become an influential international business. Not only do millions of people throughout the world rely on the industry for their livelihoods, but forestry products surround us in our daily lives. From tables to leather hides, it is almost impossible to envisage daily life without incorporating at least one forestry product. It is this dependence on forestry products that has led to the expansion of plantations all over the world. In 2004/2005 natural and plantation forestry throughout the world covered almost 4000 million hectares, accounting for 30% of the world's total land area (Department of Water Affairs and Forestry, 2007a).

Forestry refers to the use, management and downstream processing of the various forest types (Department of Water Affairs and Forestry, 2007b). The different forest types include natural forests, plantation forests and woodland savannahs (Department of Water Affairs and Forestry, 2007b). Natural forests, often referred to as indigenous forests, consist of indigenous species that have established themselves into multistrata over many decades, with little or no human influence (Department of Water Affairs and Forestry, 2007b). Plantation forests on the other hand, also known as commercial forests, are established by humans and are made up of blocks or compartments, typically of a single species, which has been selected for commercial purposes, whilst woodlands refers to anything between wooded grasslands to dense thickets (Department of Water Affairs and Forestry, 2007b).

1.2 FORESTRY IN SOUTH AFRICA

Approximately 1.1% of South Africa's total surface area is made up of commercial plantations, covering over 1.28 million ha (Department of Water Affairs and Forestry, 2007a). The higher rainfall areas of KwaZulu-Natal and Mpumalanga together

account for almost 80% of the country's total plantation area, approximately 41% and 38% respectively (Department of Water Affairs and Forestry, 2007a). Most of this land, 59%, resides in the hands of large corporate forest owners such as Sappi and Mondi (Department of Water Affairs and Forestry, 2007a). Although there are a number of smaller ventures, independent commercial farmers account for less than 28% of the country's land utilised by forestry (Department of Water Affairs and Forestry, 2007a).

The forestry industry is one of the larger providers of rural employment in South Africa. Workers in the plantation sector number over 75 000 whilst downstream the forest products sector is the fourth largest manufacturing division in the country (Tewari, 2001). The forestry sector, as a whole, provides employment to approximately half a million South Africans, a figure that is expected to increase as South Africa moves deeper into the 21st century (Jones, 1994).

The forestry industry has a large positive effect on South Africa's economy. During 2005, sales from commercial plantations reached just under R5 billion, whilst sales from forestry products came to over R15 billion (Department of Water Affairs and Forestry, 2007a). Forestry also contributes favourably on South Africa's balance of trade. In 2005 forestry product exports totalled R9.9 billion, whilst imports were considerably lower, at R8 billion (Department of Water Affairs and Forestry, 2007a).

South African plantation forestry comprises mostly exotic species from three genera: *Pinus*, *Eucalyptus* and *Acacia*. *Pinus* species account for more than 53% of the total land under plantations, whilst *Eucalptus* species account for over 37%. *Acacia* species, of which the majority is black wattle, cover a considerably smaller area, accounting for just over 8% of the total plantation area (Department of Water Affairs and Forestry, 2007a).

1.3 BLACK WATTLE INDUSTRY IN SOUTH AFRICA

1.3.1 History

Black wattle, *Acacia mearnsii* (de Wild.), was introduced to South Africa from Australia by Charles and John van der Plank in 1864 (Sherry, 1971). The first black wattle plantings were carried out in the district of Camperdown in the province currently known as KwaZulu-Natal where it was originally used to supply firewood, shelterbelts and shade for livestock (Dunlop and MacLennan, 2002).

In 1884 Australian black wattle bark started to fetch high prices in London for its tannin extract (Dunlop and MacLennan, 2002). South African farmers, recognising the potential for increasing their income, decided to assess the quality of the tannin from the bark of South African-grown black wattle (Dunlop and MacLennan, 2002). In 1888 the results obtained from these tests concluded that the South African grown black wattle bark was rich in vegetable tannins, which were of sufficient quality to be used to tan hides for the leather trade (Dunlop and MacLennan, 2002). This initiated a countrywide increase in black wattle plantations for the production of bark for the export market.

The South African bark industry grew rapidly up until the early 1960's. The resulting expansion of the South African black wattle plantations peaked at 300 000 hectares in 1960 (Beck, 1999). This peak was mainly due to the demand for leather products such as saddles and boots required by the soldiers of World War 2 (Dunlop and MacLennan, 2002). After this war ended the demand gradually decreased such that the area covered by black wattle plantations had shrunk to approximately 112 400 ha by the year 2002 (Department of Water Affairs and Forestry, 2003).

Today, black wattle is not only valued for its fuel and tannin production, but is also used for mine timber, parquet blocks, hardboard, rayon, and in recent times, it has become popular as a source of high quality pulp (Sherry, 1971; Beck, 1999). It is estimated that in South Africa the wattle industry is responsible for the production of 42 856 t of mining timber; 416 098 t of paper; 123 104 t of pulpwood; 242 561 t of wood chips; 27 778 t of charcoal; 81 692 t of firewood and 21 926 t of other by-

products per year (Department of Water Affairs and Forestry, 1999). New uses are continually being identified and developed, making black wattle one of the important components of the commercial forestry industry in South Africa today.

1.3.2 Black wattle characteristics and growth habitat

Black wattle belongs to the *Acacia* genus, which forms part of the Leguminosae family. The term leguminous means that *Acacias* have seed pods and have the ability to fix nitrogen from the air in their roots (Boland *et al.*, 1997). It is this nitrogen fixing ability, together with a thick root system that makes black wattle suitable for its use in combating soil erosion (Beck, 1999).

The leaves of black wattle are compound bipinnate, while the inflorescences form fragrant pale yellow clusters which develop into legumes that house the smooth, elliptical black seeds (Boland *et al.*, 1997). Copious numbers of seed are produced which can lie dormant for many years, retaining much of their moisture to allow growth to occur when conditions turn favourable (Sherry, 1971). It is this characteristic that is of great concern to the South African forestry industry, because it has been recognised that black wattle is one of the country's major invasive species (Pieterse and Boucher, 1997).

There are a number of growth characteristics that make black wattle appealing to the South African forestry industry. Black wattle trees grow rapidly, are extremely hardy and produce wood with good pulping properties (Dunlop *et al.*, 2000a). The trees are frost tolerant, grow in a wide range of temperatures, ranging from a mean of 0 ^{o}C in the coldest months to a mean of 28 ^{o}C, and grow at a wide array of altitudes and rainfalls (Boland *et al.*, 1997).

Whilst black wattle is able to grow in a wide range of topographical conditions, the South African climatic and topographical conditions make it difficult for the black wattle to perform optimally as a plantation species. The altitude of the landscape varies greatly throughout the country when compared to black wattle's native Australia. Only 7% of South Africa's total land area receives enough rainfall for intensive black wattle forestry (Denison and Kietzka, 1993). As a result, black wattle

plantations in South Africa are limited to the province of KwaZulu-Natal and the southern areas of the province of Mpumalanga (Figure 1.1). As the area suitable for afforestation is limited, increased demands for black wattle can only be met if there is an increase in productivity from the existing afforested land area (Beck *et al.*, 2003b).

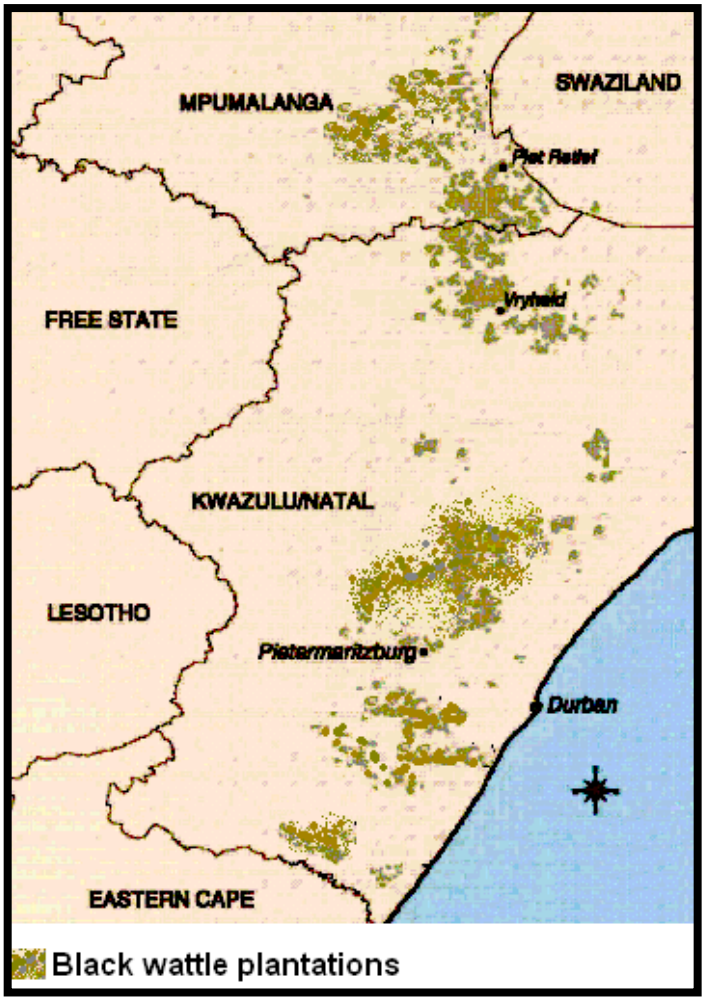

Figure 1.1 Distribution of black wattle plantations in South Africa. (Dunlop and MacLennan, 2002)

1.4 BLACK WATTLE TREE IMPROVEMENT

1.4.1 Introduction

Black wattle improvement has been practiced in South Africa for over 50 years (Dunlop and MacLennan, 2002). Osborn observed extensive phenotypic variation amongst trees that he had planted in 1927. He noted that some individuals showed

good vigour, form and resistance to disease. These early observations indicated that a tree improvement programme was a possibility and led to the appointment of Dr J. Philp by the Department of Forestry to work on wattle genetics (Dunlop and MacLennan, 2002). Dr Philp, together with Mr S.P. Sherry, then set up the Government Wattle Genetics Station at Sweetwaters in the province, which is now referred to as KwaZulu-Natal (KZN) (Dunlop and MacLennan, 2002). This station managed 40 ha of land planted predominantly with black and green wattle (*A. decurrens*) and their hybrids, but also included some other *Acacia* species (Dunlop and MacLennan, 2002).

In 1947 the Wattle Research Institute (WRI) was established as a partnership between wattle growers, the South African Government and the University of Natal. Its mandate was to improve black wattle's growth characteristics, so the genetic work that was being carried out at Sweetwaters was integrated into the new institute (Dunlop and MacLennan, 2002).

Before a comprehensive breeding programme could be developed and implemented, it was necessary to study the fundamental mechanisms surrounding the growth and reproduction of black wattle (Philp and Sherry, 1948; Dunlop and MacLennan, 2002). Thus, early research focused on pollination mechanisms and seed germination rates (Dunlop and MacLennan, 2002). More specifically, the degree of selfing and cross pollination was studied, together with experiments into hybridisation with other wattle species. At this time the researchers proposed that chromosome doubling of diploid (2n=2x) lines to produce autotetraploids (2n=4x) would result in a substantial increase in volume. Therefore, diploid seed was treated with colchicine to produce tetraploids (Dunlop and MacLennan, 2002). It was this early research that provided fundamental knowledge of the sexual mechanisms of black wattle and formed the back-bone of the current black wattle tree improvement programme (Philp and Sherry, 1948; Dunlop and MacLennan, 2002).

During 1984 the WRI became the Institute for Commercial Forestry Research (ICFR) to additionally conduct forestry research for the benefit of commercially grown eucalypts and pines (Dunlop and MacLennan, 2002). Although much of the eucalypt

and pine breeding is carried out within commercial companies, the black wattle breeding programme is managed solely by the ICFR (Jones, 2002).

1.4.2. Breeding characteristics

In the past, tannin was the major product of black wattle and hence the early improvement programme focused mainly on the improvement of black wattle bark for tannin production. Other characteristics such as frost tolerance and disease resistance were also addressed during those times (Sherry, 1971; Jarmain and Lloyd-Jones, 1982). Today, black wattle timber has been identified as a good source of pulp for the pulp and paper industry, which has resulted in a shift of focus in the black wattle improvement programme to include these market needs (Dunlop *et al.*, 2000b). Thus, the current black wattle improvement programme has prioritised the improvement of pulp and paper properties such as fibre yield and quality (Dunlop and MacLennan, 2002). Although traits for frost and disease tolerance, such as tolerance to the disease gummosis, remain important qualities for improvement, growth remains a top priority to the industry (Dunlop and MacLennan, 2002). The primary aim of the improvement programme is thus to improve fibre yield and quality whilst maintaining current bark quality levels (Dunlop and MacLennan, 2002).

In recent years, black wattle's invasive nature has been recognised as a problem, leading to its classification as a top alien invader (Pieterse and Boucher, 1997). The ICFR has thus extended the breeding programme to include the control of seed production. Two major strategies are currently under investigation (Beck, 2004). The first strategy is to develop triploid (3x) black wattle, which will be largely sterile due to the variable chromosome pairing associations during meiosis, resulting in unbalanced, infertile gametes, thereby curbing viable seed production (Chaudhari and Barrow, 1976; Ramsey and Schemske, 1998; Beck, 2004). In order for this strategy to be successful, the triploids must retain the growth rate and timber characteristics of the current diploid lines.

The second strategy to curb the prolific seed production of black wattle is to produce sterile trees using gamma irradiation. Gamma irradiation causes DNA damage, oxidative base damage, single-strand breaks and double-strand breaks (Rothkamm

and Lobrich, 2003). This damage affects the normal progression of meiosis, thereby reducing viable gamete formation and subsequent seed production. The correct irradiation dosage needs to be determined so that suitable DNA damage occurs, thereby reducing seed production, without much negative effect on vegetative growth.

1.4.2. Breeding strategies

1.4.2.1 Introduction

A breeding strategy, in a forestry context, is an overview of the management of genetic improvement of a particular tree species that is utilised in plantation forests (Eldridge *et al.*, 1997). Most breeding strategies comprise a breeding and a production component (Zobel and Talbert, 1984). These two components are run concurrently, where possible, in an attempt to optimise gains made over a single improvement generation (Hettasch *et al.*, 2006). The breeding component of a breeding strategy involves the maintenance, management and improvement of a broad-based genetic pool, while the production component involves the production of improved genetic material to the grower, in order to maximise economic gain (Hettasch *et al.*, 2006). Both the breeding and production components of a breeding strategy rely upon isolated plantations of trees that are assumed to comprise of genetically superior individuals. These plantations are known as seed orchards (SO) (Hettasch *et al.*, 2006).

Seed orchards (SO) are isolated to prevent pollen contamination and intensively managed to supply improved seed frequently, abundantly and easily (Hettasch *et al.*, 2006). A SO found in the breeding component of a breeding strategy is called a breeding SO (BSO). Similarly, seed orchards found in the production component are called production SOs (PSO). BSOs make up the main breeding group, which is larger, less genetically advanced and less intensely managed than the nucleus breeding group, which is made up of PSOs (Dunlop and MacLennan, 2002; Hettasch *et al.*, 2006). PSOs may comprise of individuals grown from improved seed as well as cloned trees, whilst BSOs are almost always seedling orchards due to their large size and the prohibitive expense of cloning trees. All SOs rely on a process whereby

certain individuals are favoured for reproduction because of the genetic traits they display, called selection (Hettasch *et al.*, 2006). It is selection that enables a seed orchard to be properly established.

Selection is the primary tool used in breeding programmes to improve the genetic material and realise genetic gains (Hettasch *et al.*, 2006). Selection intensity can be adjusted by altering the selection differential. Selection differential is the term given to the deviance of a selected population's mean from that of the original population. Generally BSOs are selected at a lower selection differential than are PSOs (Hettasch *et al.*, 2006). Although selection can be carried out phenotypically, most breeding programmes utilise some form of backward selection, whereby selections are made on the parent generation based on the performance of the offspring or progeny. This performance is evaluated in trials known as progeny tests (Dunlop and MacLennan, 2002; Beck, 2004).

In order to make informed selections it is important to have an understanding of the degree to which a particular trait is passed on through to the next generation. This is known as the heritability of a trait and heritability values express the proportion of phenotypic variation that is due to genetic differences (Hettasch *et al.*, 2006). The higher the heritability values, the less of a role the environment plays in the phenotype of a trait, whilst the role of the genotype increases. Heritability can be further divided into broad-sense (H^2) and narrow-sense (h^2) heritability. Broad-sense heritability is defined as the ratio of total genetic variation in a population to phenotypic variation, whilst narrow-sense heritability is defined as the ratio of additive or allelic genetic variation to phenotypic variation (Hettasch *et al.*, 2006). Narrow-sense heritability is never higher than broad-sense heritability as narrow-sense heritability only considers variation due to additive or allelic effects, whilst broad-sense heritability not only takes this into account, but other non-additive genetic effects such as dominance and paternal or maternal effects (Hettasch *et al.*, 2006).

Understanding the combining abilities of breeding individuals makes the selection of an appropriate breeding strategy more attainable. Combining abilities can be general or specific. General combining ability (GCA) is defined as the average performance of the progeny from a particular parent compared to the population mean, whilst

specific combining ability (SCA) is defined as the performance of the progeny from a specific cross compared to the expected performance of the two parents based on their individual GCAs (Hettasch *et al.*, 2006). GCA measures additive variance, whilst SCA measures non-additive variance. Thus, when put in a breeding perspective, parents that show a high GCA, are likely to show good narrow-sense heritability and will be best managed in an open pollinated seed orchard, whilst parents that show a high SCA will be less likely to show good narrow-sense heritability and should be managed in a breeding strategy that utilises controlled pollination, i.e. pedigree breeding. Therefore when designing a breeding strategy it is important to have an understanding of the interactions between parents and progeny, so as to manage selections in the most appropriate manner.

1.4.2.2 The ICFR black wattle breeding strategy

In 1984, when the WRI became the ICFR to include research on eucalypts and pines, research and breeding of black wattle continued (Dunlop and MacLennan, 2002). Today the ICFR remains a world leader in black wattle research and breeding and whilst the lessons of the past have not been lost, new tools enable researchers to look at old problems in a new light. Since 1993 the ICFR has developed and implemented a multiple population breeding strategy (MPBS) (Figure 1.2) (Dunlop and MacLennan, 2002; Dunlop *et al.*, 2003). This MPBS utilises selections from a wide variety of populations at different levels of improvement.

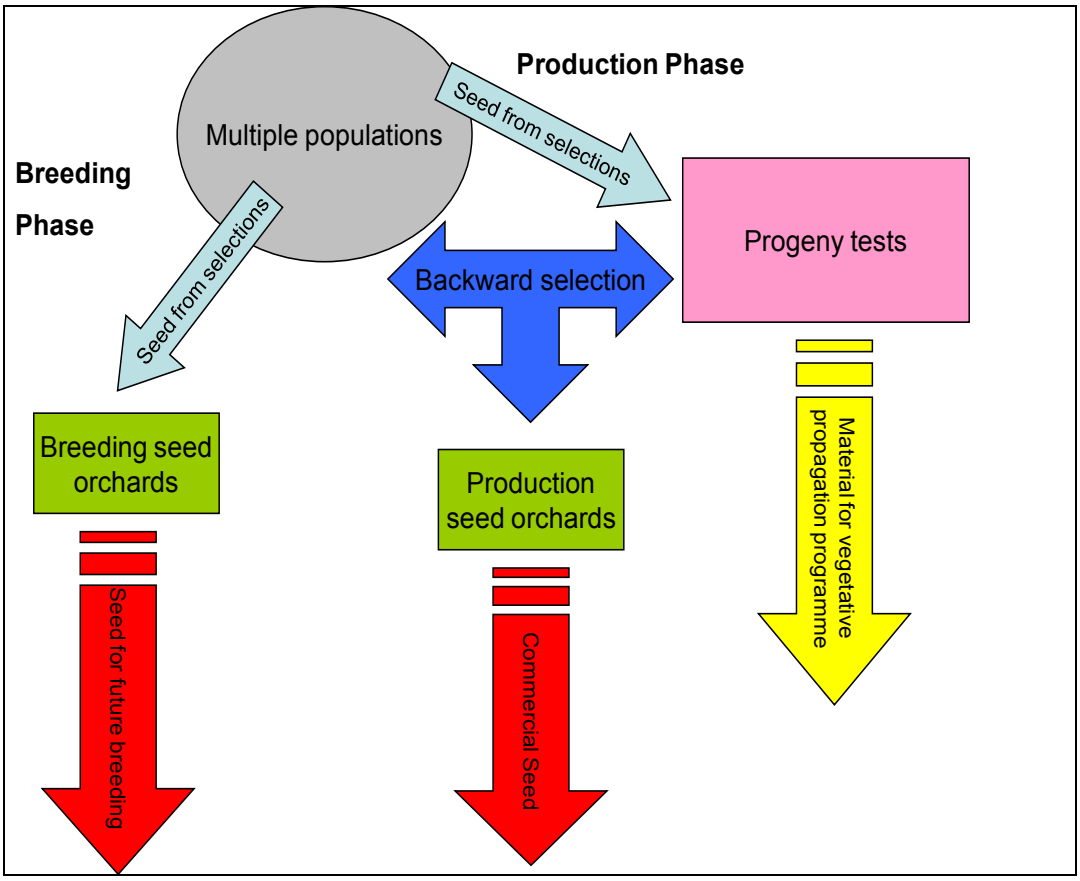

Figure 1.2 Representations of the current black wattle multiple population breeding strategy currently implemented by the ICFR (Dunlop and MacLennan, 2002).

The current ICFR black wattle breeding programme has seed from 1081 selections, made up from 1st, 2nd, 3rd and 4th generation selections together with 120 selfed seedlots. The full pedigree of these selections is known and selections have been made from commercial plantations and progeny trials (Dunlop and MacLennan, 2002). Seed, either open pollinated or from controlled pollinations, from these superior selections have been used to establish 14 PSOs and some of these families are currently being tested in the vegetative propagation project. These PSOs supply the forestry industry with improved seed for commercial plantings. BSOs have also been established for further breeding and are a subset of the original selections (Dunlop and MacLennan, 2002).

Whilst maintaining acceptable levels of bark yields, the MPBS is producing new-generation seed with improved timber quantity and quality (Dunlop and MacLennan,

2002). Other options, such as hybridisation with other suitable *Acacia* species, and the production of polyploids have been undertaken and are being re-investigated (Philp and Sherry, 1948; Moffett and Nixon, 1958; Moffett and Nixon, 1960; Dunlop and MacLennan, 2002; Beck *et al.*, 2003a; Beck *et al.*, 2005). Due to the vigorous growth and robust nature of some polyploids, this avenue of research seems exciting. Some polyploids are under investigation for a completely different reason; triploidy reduces seed vigour and in doing so, it may help reduce the invasive nature of black wattle (Beck *et al.*, 2003a; Beck *et al.*, 2005). However the success of these avenues of research depends largely on a successful clonal programme (Beck and Dunlop, 2001).

1.5 VEGETATIVE PROPAGATION

Cloning, either through macro- or micropropagation, is an important aspect in the black wattle improvement programme. Whilst traditional breeding is relatively simple and inexpensive, some non-additive genetic effects, such as dominance and maternal effects are difficult to capture through this method (Hettasch *et al.*, 2006). Apart from this, breeding programs are also limited due to the large tracts of land required for screening through progeny testing, and further, by the long life cycles of forest trees (Tomar and Gupta, 1988). Both the tree improvement programmes and the production of sterile wattle rely on the development of a successful and economically viable cloning procedure.

The genetic cloning of a superior individual can be accomplished through traditional macropropagation methods or by more recently developed micropropagation methods. Macropropagation utilises nursery techniques and is carried out *ex vitro*, whereas micropropagation utilises laboratory techniques and is carried out almost exclusively *in vitro* (Beck and Dunlop, 2001). The theory behind macro- and micropropagation are similar and it is mainly the scale of the plant material that varies.

Macropropagation methods, such as grafting, rooted cuttings and air rooting, were among the first techniques used to propagate forest trees vegetatively (Barnes and Burley, 1987). Since these early trials, tip cleft, side veneer, budding and other

grafting methods have played a significant role in commercial forestry improvement. Despite the potential advantages of grafting, these propagation methods have often been criticised and are not considered economically viable (Jones, 1994). Rooted cuttings remain a popular method of propagation and although this method is successfully used in most eucalypt hybrids grown in South Africa, the effect of plant material age on rooting restrict the use of this technique, especially in the case of black wattle (Watt *et al.*, 1995; Beck, 1999). There is also a significant lag time in this method because mature plant material is extremely difficult to root, therefore clones require extensive testing in the field before superior individuals are selected - a process that takes many years. As a result of the drawbacks of macropropagation, more research has been directed to micropropagation methods of cloning.

1.6 MICROPROPAGATION

1.6.1 Introduction

Micropropagation of forest trees is a new and developing area that is making rapid progress in the field of forest biotechnology. This technology holds such promise due to the numerous advantages it displays over conventional methods of cloning (Han *et al.*, 1997). One of the major advantages of micropropagated trees is the fact that they regain juvenile characteristics, such as rapid growth, allowing superior mature adults to be selected and then cloned (Beck, 1999). The advantages of this technology are numerous: the ability of micropropagation to rapidly produce multiple copies of a single genotype is also a major advantage, but the techniques themselves can be complex. Care must be taken to choose the right technique, a suitable explant and the correct growth media, accompanied by the correct hormones, under the optimum culture conditions, for each unique species and end result (Nehra and Kartha, 1994). Micropropagation, whilst expensive and highly intensive, may hold the key to overcoming numerous problems associated with the conventional cloning of commercial forestry species, especially with regard to traditionally hard-to-root species such as black wattle.

A small explant is excised from a previously identified, genetically superior mature adult tree; the explant is then induced, on culture medium *in vitro* under sterile

conditions, to directly or indirectly form plant organs, which in turn result in a number of genetically identical plantlets; these plantlets grow into several clones, each sharing the same genetically superior traits found in the original mature adult tree (Hettasch *et al.*, 2006). In short, clonal micropropagation is similar to traditional methods of cloning such as rooted cuttings, but is performed on a micro scale, in a controlled, sterile environment.

There are two major groups of clonal micropropagation methods for elite tree production (Jones, 1994). The first is direct organogenesis. Here organs such as roots and shoots are induced on media directly from the explant (the types of explant used are numerous and buds may be harvested from many areas of the selected plant) with no intervening callus step, thereby minimising the possibility of somatic mutations (Ryynänen and Aronen, 2005). On the other hand, indirect organogenesis makes use of a callus culture step. The explant is placed on culture medium and induced to form callus through the use of various chemicals and hormones. This callus step places the explant under stress and heightens the probability of somatic mutations (Ryynänen and Aronen, 2005). From here the callus is transferred to a new medium where it is induced to form particular organs such as shoots or roots. A method that forms part of the indirect organogenesis group, but is of significant importance is somatic embryogenesis. This method differs from simple organogenesis through the formation of somatic embryos that have the ability to germinate instead of forming other organs such as shoots (Maillot *et al.*, 2006).

Indirect organogenesis is well suited to attaining genetic transformation through micropropagation. The state of constant cell division during the undifferentiated stem callus stage of indirect organogenesis is the reason these methods are more suited to genetic transformation techniques than to traditional cloning (Ryynänen and Aronen, 2005). The constant state of mitotic division increases the possibility of somatic mutations arising, and whilst when transforming a clone this fact is not important, when trying to conserve the genotype of a particular superior individual, then it is an expensive disadvantage (Ryynänen and Aronen, 2005). Whilst DNA fingerprinting can be used to screen the integrity of the genome, this technique is expensive and adds an additional cost on an already expensive cloning technique (Caetano-Anollés, 1998).

Direct regeneration or direct organogenesis does not make use of a stem callus phase and hence the likelihood of somatic mutations is greatly reduced (Ryynänen and Aronen, 2005). This method is therefore the obvious choice when attempting to clone a mature, genetically superior individual, whilst conserving its genotype, as it reduces the need for expensive screening processes such as DNA fingerprinting.

1.6.2 Explant selections

The size, position and type of explant offer a series of practical solutions to the many problems associated with clonal micropropagation (Majourhat *et al.*, 2007). Ultimately, explant choice depends on the species being worked with, and the field requirements of the parent material. An explant excised from a healthy plant, with mobilised nutrients for good growth will benefit the micropropagation process (Renau-Morata *et al.*, 2005). Also particular species traits, such as how well the species takes to tissue culture, and whether the species is susceptible to internal contaminants, such as viruses, are very important to consider when selecting a suitable explant. The environment and growth conditions experienced by the parent plant are also very important factors to consider and some suitable explant choices may be ruled out by the conditions of the parent material. For instance, the use of coppice material, a common explant that shows better elongation and a greater root potential then other explants excised from the crown, may be ruled out, simply because the field conditions of the parent material do not allow for the felling of the identified individuals (Bonga and von Aderkas, 1992). Whilst the importance of field circumstances cannot be ignored when choosing a suitable explant, the specific nature of the species under study remains the most important consideration. Species traits of significant import are: susceptibility to internal contamination; ability to thrive under tissue culture conditions; and the ability to sprout and coppice. These traits must be investigated at length, before an explant selection for a particular species is made.

Species that show a resistance to internal contamination allow a greater freedom in explant choice when compared to more contamination-susceptible species. More resistant species allow for the use of large explants, which are easier to work with

and are more likely to thrive under tissue culture conditions. An example would be the use of stem micro-cuttings in the micropropagation of *Eucalyptus* hybrids (Watt *et al.*, 1995). However, some species, such as black wattle, are more susceptible to internal contamination and this presents a more complex problem (Beck, 1999). The distribution of these pathogens is not uniform within the plant and it has been proposed that virus particles decrease in number as the apical dome is reached (Bonga and von Aderkas, 1992). Therefore, for a species that is highly susceptible to internal contamination, the apical meristem is a practical solution to this problem. However due to the small size of the apical meristem it is only suitable for use in species that take well to tissue culture conditions (Bonga and von Aderkas, 1992).

Unfortunately, many species do not take well to tissue culture and black wattle is one such species (Beck, 1999). For these species, a larger explant is required as it is difficult to establish successful cultures from such small explants (Bonga and von Aderkas, 1992). Alone this problem is easily resolved, but in the case of species that are highly susceptible to internal contamination, it presents an intriguing dilemma. Whilst easier to propagate, larger explants have an increased level of contamination (Bonga and von Aderkas, 1992). One possible explant for problematic species is the shoot region called the shoot apex. The shoot apex is the name given to the apical meristem together with the upper layers of leaf primordia that occur just below the apical dome (Dodds and Roberts, 1985). This shoot apex region is more suited to use in tissue culture than the apical meristem, because it makes use of a larger explant, and whilst the shoot apex region is more likely to contain pathogens, it also provides an explant area that has a large reduction in internal contamination levels (Beck, 1999).

Coppice material provides many advantages and should be used whenever the species traits and field circumstances allow. Coppicing has the advantage of increasing juvenile characteristics of adult material, which in turn increases the reactivity plant material shows to tissue culture hormones (Beck, 1999). Explants excised from coppice material also reduce the levels of contamination *in vitro*, illustrating an important point; with the right explant choice the levels of potentially damaging chemicals needed to sterilize the explant can be reduced (Beck, 1999).

1.6.3 Sterilant selections

When selecting a sterilant, or more accurately, a sequence of sterilants, it is not only important to minimize the damage to the explant, but also to reduce the levels of surface contamination during culture. It is essential to the success of the whole regeneration process that no bacteria or fungi are present on or in the explant, because the culture medium is an ideal growth medium for many micro-organisms (Beck, 1999). If these micro-organisms are introduced into the culture media, they quickly infest the desired explant because they are able to reproduce and grow rapidly (Beck, 1999). To avoid this microbial invasion, surface sterilization is undertaken to remove any contaminants that may be living on the surface of the harvested explants.

The ideal sterilant should do the least amount of damage, whilst reducing contamination to a minimum level. In a contaminant-free environment or when using explants cultured *in vitro*, the explants would simply be rinsed and no damage would be incurred. Unfortunately this is not usually the case and a number of factors influence the strength of sterilants required. Specific species traits and previous protocol selections strongly influence the strength of sterilants required. Some species are more susceptible to contamination than others and these species require a more vigorous and damaging sterilisation sequence (Rajesh and Anil, 2006). Depending on the initial aims of the cloning process, and the method that is selected, a certain incidence of contamination may be deemed acceptable. If the acceptable level of contamination is lower than normal, for example when using a bioreactor system, then the sterilisation sequence must be more vigorous, and the resultant higher mortalities caused by sterilisation must be tolerated (Kim *et al.*, 2003). Alternatively an extra contaminant screening stage must be incorporated into the cloning process. Explant selection, as alluded to earlier, also has a large impact on the sterilisation process. Larger explants generally carry more contaminants, but can withstand higher sterilant-abuse than smaller ones (Bonga and von Aderkas, 1992). Therefore, explant size, together with plant and explant type strongly influence sterilant selection.

Surface contamination levels and hence the strength of sterilants required, are largely dictated by environmental factors, and the age and physiological status of the parent material (Siril and Dhar, 1997; Bhatt and Dhar, 2004). Older plants have been exposed to contaminant microbes for longer periods than younger material and hence an explant from a mature parent plant requires a vigorous sterilisation process. Likewise parent material from a sterile *in vitro* environment will require a gentler sterilisation than material from a nursery. Similarly certain climates (usually hot, damp climates) promote bacterial and fungal growth, which in turn necessitate a stronger sterilisation process (Etchells *et al.*, 1973). The abundance of fungi and bacteria has been shown to vary according to season, indicating that the time of year also effects the sterilisation process (Matheron *et al.*, 2005; Ipsilantis and Sylvia, 2007).

1.6.4 Culture makeup and conditions

The responses of the explant in tissue culture are affected by the type of supporting medium, the temperature of incubation, the wavelength, intensity and period of lighting conditions and the nutrient and growth hormone levels (Beck, 1999). These factors, both physical and chemical, combine in complex interactions and may have positive or negative effects on the growing explants.

The selection of chemical and hormonal factors plays a key role in the success or failure of any micropropagation technique. This selection does not only depend on the specific species traits and previous protocol selections, but also on the stage of development of the regenerating explant (Beck, 1999). For instance, the hormones required to induce callus from an explant vary from those required to induce shoots and these in turn vary from those required to induce roots. As a result, there is a startlingly large array of possible choices and combinations available when selecting the hormonal makeup of any one clonal micropropagation process.

Whilst many media have been used in the micropropagation of *Acacia* species, such as: Gamborg medium (Gamborg *et al.*, 1968); Schenk and Hildebrandt medium (Schenk and Hildebrandt, 1972); Quoirin and Lepoivre medium (Quoirin and Lepoivre, 1977) and Woody Plant Medium (WPM) (Lloyd and McCowan, 1981),

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) is the most commonly used (Beck and Dunlop, 2001). Various combinations of cytokinins and auxins can be added to the medium to induce different organs. For the *Acacia* genus, combinations of benzyladenine (BA) and α-naphthaleneactic acid (NAA) for shoot initiation, gibberellic acid (GA) for shoot elongation and various concentrations and combinations of auxins for root induction have been recommended (Beck and Dunlop, 2001).

Other key components of most micropropagation techniques include the particular culture conditions to which the growing explant is exposed. Ideal temperatures vary according to the species, whilst ideal day and night temperature differences vary according to the particular organ being induced. Similarly, ideal light periods differ according to species, whilst light intensity dictates the growth of the explant and the induction of organs can be influenced by different wavelengths of light (Appelgren, 1991; Nhut *et al.*, 2003).

The success of any micropropagation technique requires a fine balance between culture medium, growth conditions and plant physiology. By constantly altering the culture and growth conditions, plant growth responses can be manipulated as required and a new plantlet can be produced that is genetically identical to the mother plant; however, it is important to remember that the *in vitro* micropropagated plantlet may not possess the required attributes to survive in an ever-changing and harsh external environment.

1.6.5 Acclimatisation

After any successful micropropagation technique, whereby an explant has been induced to form a complete growing plantlet, it is crucial to move the plantlet gradually from the controlled, sterile conditions experienced *in vitro* to the harsh environmental conditions experienced *ex vitro*. This gradual stepwise process is known as acclimatisation or 'hardening off' (Hazarika, 2003; Pospísilová *et al.*, 2007).

Acclimatisation is necessary to ensure that a large proportion of plantlets survive the transition from *in vitro* to *ex vitro* (Hazarika, 2003). This transition is fatal to many

micropropagated plants because the plants need time to adapt their physiology to the external conditions. In the greenhouse and in the field the plants are exposed to much lower air humidity, higher light levels, more air turbulence and a wider range of temperatures (Hazarika, 2003; Pospísilová *et al.*, 2007). In addition, during micropropagation, nutrients are provided in a form that is easy for the plant to assimilate for growth, which further illustrates why many plants do not survive this phase of the micropropagation technique.

The stringently controlled environment experienced by a micropropagated plantlet results in physiological abnormalities and these abnormalities must be reduced during the acclimatisation period, before the plantlet is transplanted *ex vitro* (Pospísilová *et al.*, 2007). During acclimatisation, the control of environmental factors, such as humidity and temperature, is slowly and systematically reduced until, after a period of weeks or even months, the micropropagated plant may be planted out into the natural environment. This period is crucial, not only to reduce the stresses imposed on a plant that is not adapted to a harsh external environment, but also to allow the plant time to establish itself and grow larger leaves and roots that will be key for its continued survival (Hazarika, 2003; Pospísilová *et al.*, 2007).

A micropropagation technique can not be labelled successful until the micropropagated plants are able to establish themselves *ex vitro*, thus it is important to remember that acclimatisation is not a separate activity, but rather an important step in a larger process, termed micropropagation.

1.7 APPLICATIONS OF MICROPROPAGATION IN BLACK WATTLE

Currently there are two main reasons for research into the micropropagation of black wattle in South Africa and these are closely linked. The first and most financially important application is the ability micropropagation technology gives the breeder to select and propagate mature tissue, which in turn allows for better capture of selected traits such as high volume, good stem form and high pulp yields (Jones and van Staden, 1997). The second and more environmentally focused application deals with the highly invasive nature of black wattle. It is hoped that research into the sterilisation of black wattle will be successful, and if this is the case, then foresters

will have to rely on clonal propagation to supply material for their growing operations (Beck and Dunlop, 2001). However, in order for sterile black wattle to be viable, the genetic gains through clonal forestry must be large. It is in this way that the economic and environmental applications are linked.

The utilisation of micropropagation in black wattle is still in its infancy when compared to other species. The little research that has been performed on this species has been rewarded with varying degrees of success (Table 1.1) when compared to other commercially viable *Acacia* species. The fact that this technology has not been used and researched as extensively as other *Acacia* species is concerning because the eventual deployment of sterile black wattle may depend on the ability to successfully micropropagate adult material. Whilst protocols have been developed for the successful micropropagation of *in vitro* grown explants, techniques utilising mature plant material have only resulted in shoots, showing limited success (Table 1.1). The aims of this study were therefore:

1. To investigate the effects of different sterilisation agents and exposure times on cultured *Acacia mearnsii* shoot apex contamination levels.

2. To test the effects of harvest season and individual mother plant on shoot initiation during shoot apex culture of *Acacia mearnsii*.

3. To investigate the effects of physical and hormonal factors on shoot elongation of *Acacia mearnsii* during shoot apex culture.

Table 1.1 Summary of micropropagation methods utilised in black wattle (*Acacia mearnsii*), the explants used and the results obtained.

Micropropagation method	Explant	Results obtained	Reference
Direct organogenesis	*In vitro* grown nodes	Shoots, roots and acclimatisation	(Beck *et al.*, 1998a)
Direct organogenesis	Nodes from coppice	Shoots	(Beck *et al.*, 1998b)
Direct organogenesis	*In vitro* grown meristems	Shoots	(Beck *et al.*, 2000)
Direct organogenesis	Meristems from coppice and adult material	Shoots	(Beck *et al.*, 2000)
Direct organogenesis	Nodes from 9 month old seedlings	Shoots	(Correia and Graça, 1995)
Direct organogenesis	*In vitro* grown nodes	Shoots	(Borges Júnior *et al.*, 2004)
Direct organogenesis	*In vitro* grown shoot tips	Shoots and roots	(Huang *et al.*, 1994)
Indirect organogenesis	*In vitro* grown hypocotyls and cotyledons	Callus, buds and roots	(Quoirin *et al.*, 1998)
Direct organogenesis	*In vitro* grown nodes	Shoots and roots	(Quoirin *et al.*, 2001)

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CHAPTER 2

SCREENING OF TOPICAL STERILANTS FOR SHOOT APEX CULTURE OF *ACACIA MEARNSII*

ABSTRACT

The surface sterilisation procedure is of integral importance to any micropropagation technique. This process should do the least amount of plant damage, whilst reducing microbial contamination to an acceptable level. The objective of this research was to investigate alternative sterilisation agents to the dangerous chemical, mercuric chloride ($HgCl_2$), and to determine the effect of sterilant exposure time on shoot apex culture of *A. mearnsii*. Explants were cultured on MS medium supplemented with 2.0 mg L^{-1} BA and monitored for 21 d for signs of contamination and shooting. Household bleach (NaOCl), diluted 1:3 in water, resulted in decontaminated explant levels not significantly different to the 0.2% $HgCl_2$ treatment and resulted in shoot development levels significantly higher than the 0.2% $HgCl_2$ treatment. There was no significant effect of sterilant exposure time on decontaminated explant levels, whilst the shortest exposure time resulted in significantly higher shooting levels than the other two time periods tested.

2.1 INTRODUCTION

Clonal forestry through tissue culture may provide a superior alternative to traditional cloning techniques (Han *et al.*, 1997). Tissue culture techniques have been attempted on a large range of tree species. The success of such attempts depends on the complex interactions between explant, plant growth regulators, culture conditions and genotype (Nehra and Kartha, 1994). Although substantial research has been conducted on *Acacia* species, resulting in varying degrees of plantlet regeneration, micropropagation research on *A. mearnsii* is still limited (Beck, 1999).

Numerous techniques are currently being utilised for the micropropagation of forest trees. Tissue culture methods for elite tree production include the use of buds, shoots and nodal explants to induce plant regeneration (direct organogenesis), the use of callus cultures to induce plant regeneration (indirect organogenesis) as well as artificial embryo production from such callus cultures (somatic embryogenesis) (Jones, 1994). The state of constant cell division during the undifferentiated stem callus stage of the latter two techniques increases the possibility of somatic mutations arising (Ryynänen and Aronen, 2005). Somatic mutations can be an expensive disadvantage when trying to conserve the genotype of a particular superior individual; hence, in these cases it is preferable to utilise direct organogenesis.

Meristem culture has been utilised extensively across a wide array of plant and crop species (Beck, 1999). The shoot apical meristem, a dome of totipotent cells at the tip of the shoot, allows for the production of contaminant-free plants (Bonga and von Aderkas, 1992; Nehra and Kartha, 1994). The meristem region makes for a very small explant and for many tree species, a larger explant is required, because it is difficult to establish successful cultures from such small explants (Bonga and von Aderkas, 1992). The shoot apex is the name given to the apical meristem, together with the upper layers of leaf primordia, that occur just below the apical dome (Dodds and Roberts, 1985). This shoot apex region may be more suited to use in tissue culture than the apical meristem because it makes use of a larger explant. Whilst the shoot apex region is more likely to contain pathogens than the meristem region alone, it still provides an explant area that has a large reduction in internal contamination levels when compared to most other explants (Beck, 1999).

Explant material grown *ex vitro* is exposed to a countless array of possible tissue culture contaminants and microbes. As prevention and avoidance of contamination is crucial in achieving successful plant tissue culture, it is important to consider a suitable sterilisation procedure (Beck, 1999). The ideal sterilisation process should do the least amount of plant damage, whilst reducing microbial contamination to an acceptable level.

Although a suitable sterilisation process has been described for use on black wattle explants, the process makes use of $HgCl_2$, an environmentally dangerous chemical (Saha, 1972). Furthermore heavy metals negatively affect plant growth and development and have been shown to inhibit hypocotyl length (Mor *et al.*, 2002). Thus there is an essential need to improve the topical sterilisation process of *A. mearnsii* explants.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Forty two-year-old black wattle trees growing in 50 L black cultivation bags were utilised to provide explants. The trees were kept under 20% shade cloth at the Institute for Commercial Forestry Research (ICFR) nursery and were watered regularly via an automated irrigation system to prevent the bags from drying out. Trees selected for explant harvesting appeared healthy, displayed vigorous growth and abundant green foliage. All explants were harvested from the trees during the spring (August to November) of 2003.

2.2.2 Selection and collection of shoot apexes

Healthy young shoots comprising branched sections with up to six shoot apexes were selected, resulting in a greater chance of mitotic division and thereby increasing the chance of regeneration during micro-propagation. These shoots were carefully removed from a bagged tree by hand and placed in a sterile petri dish during mid-morning.

Shoots from several different trees were harvested and placed in one sterile container. Depending on experimental needs 60, 120 or 180 shoot apexes were harvested on each collection day.

2.2.3 Topical sterilisation

The harvested shoots were taken in sterile petri dishes directly from the nursery to the ICFR laboratory where they were exposed to a topical sterilisation process to remove any surface contaminants. All collected shoots underwent the following topical sterilisation process:

Step 1 Thoroughly wash under running tap water
Step 2 Surface sterilise in 70% ethanol for 1 min
Step 3 Soak in 0.2% Benlate® (benomyl 50% a.i.; Du Pont de Nemours, South Africa) for 10 min
Step 4 Rinse in distilled water
Step 5 Screen topical strerilants as per list below in Table 2.1
Step 6 Rinse in distilled water
Step 7 Soak in 500 mg L^{-1} ascorbic acid for 15 min

The following list comprises the sterilants screened for suitability for shoot apex culture of black wattle and the particular concentrations at which they were screened:

Table 2.1 Topical sterilants screened, showing household trade name, active ingredient and active ingredient concentration.

Tradename	Active Ingredient	Concentration (% Active Ingredient)
Mercuric chloride	$HgCl_2$	0.2
HTH	$Ca(OCl)_2$	7
Jik 25%	NaOCl	0.875
Jik 50%	NaOCl	1.75
Jik 75%	NaOCl	2.625

For each of these sterilants three different exposure times were investigated; 10 min, 20 min and 30 min. For each exposure time, 60 shoot apexes were sterilised.

2.2.4 Dissection, culture media and growth conditions

After the shoots had been topically sterilised, they required dissection to isolate the shoot apexes (Figure 2.1). Dissection was undertaken on a laminar flow bench with a sterile scalpel. The shoot apexes were carefully placed on culture medium using sterile tweezers.

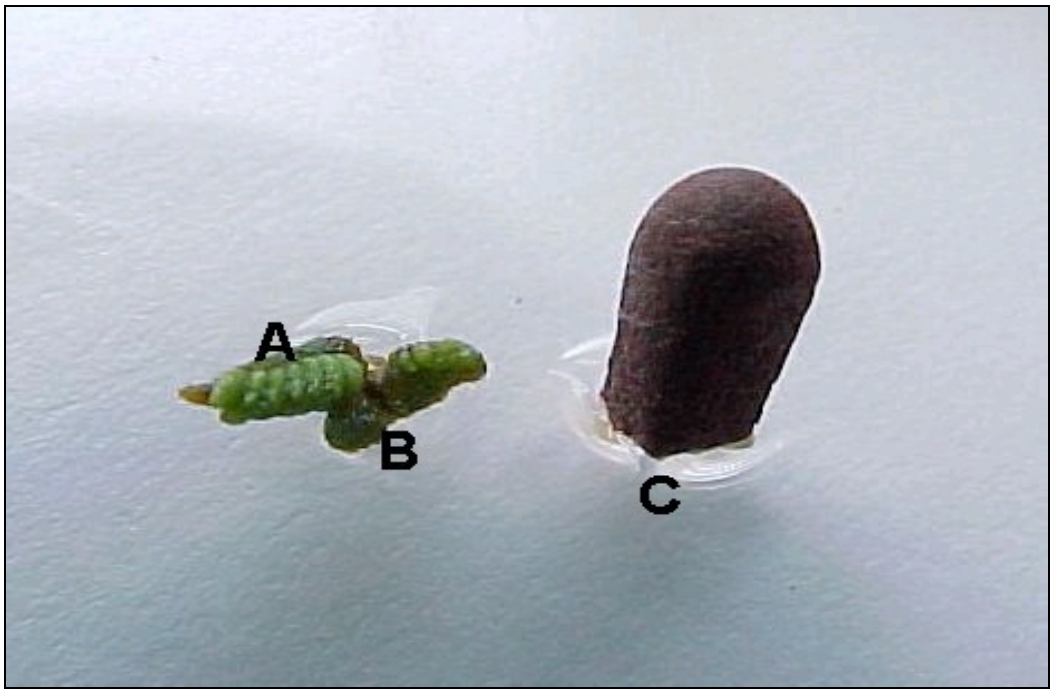

Figure 2.1 Size of *A. mearnsii* shoot apex relative to a match head (C). The meristematic region (B) is located between two leaf primordia (A).

Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} BA was chosen as the shoot-initiation medium as it was shown to be successful with *A. mearnsii* micropropagation in previous studies (Murashige and Skoog, 1962; Beck *et al.*, 1998; Beck, 1999; Beck *et al.*, 2000). The medium was supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} Myo-inositol, altered to a pH of 5.8. Gelrite, 3 g L^{-1} (solidifying agent) was added prior to the medium being autoclaved (at 121°C and 103 kPa for 20 min). Fifteen plates (65 mm sterile plastic petri-dishes from Labotec), each containing four shoot apexes, were used for each treatment and sealed using parafilm. All cultures were

maintained at a temperature of 25 ± 2°C, incubated under continuous light (23 µmol m^{-2} s^{-1}) with a photoperiod of 16 h light / 8 h dark. Cool white fluorescent tubes provided the light source. Recipes of all basal media tested are listed in the Appendix 1 and all culture protocols were carried out using aseptic technique.

2.2.5 Monitoring and quantification of results

In order to quantify the effect of each topical sterilant and exposure time, it was necessary to place the explants on culture medium to initiate shoots. Treatments were screened not only for contamination levels, but also for the most successful shooting rate. The apexes were monitored on a weekly basis for 21 d. On each occasion that an explant became contaminated, the surviving explants were transferred to a new plate. Three weeks after initial placement on culture medium, the number of decontaminated explants (free of visible microbial growth), together with the number of shooting explants in each plate was recorded. Shooting was defined by a visual doubling in length of the explant, excluding callus growth.

2.2.6 Statistical analysis

Statistical analysis was carried out using Genstat Version 9.1. It was necessary to transform the data before analysis since it was count-data and the range of the data was invariably greater than the mean (Rayner, 1969). Data was transformed according to the following linear transformation: $y = x + 10$; where x is the original data and y the transformed data. A two-way (sterilant and exposure time) ANOVA without blocking was performed on the transformed data and the results were recorded. This analysis was performed on both data sets. Two linear regressions were performed, one between exposure times and transformed shooting data, and another between transformed decontaminated explant data and transformed shooting data.

2.3 RESULTS

Results are shown without transformations, unless specified, as the linear transformation did not alter significance levels, only the coefficient of variation. The results of both the two-way ANOVA analyses (decontaminated- and shooting data) showed differing levels of significance (Table 2.2). Subsequent analysis of shooting counts, with decontaminated counts run as a covariate, showed this covariate effect not to be significant (Table 2.2). Therefore, no further results have been shown adjusted for any covariate.

Table 2.2 Summary of the analyses of variance for decontaminated-, shooting- and shooting counts adjusted for the covariate, decontaminated counts. All data was transformed by linear transformation prior to analysis and least significant differences of means (l.s.d.) are listed at the 5% level.

Treatment Interactions		**Decontaminated**			**Shooting**			**Shooting with covariate**		
Source of variation										
	d.f.	F pr.	m.s.	l.s.d.	F pr.	m.s.	l.s.d.	F pr.	m.s.	l.s.d.
Sterilant	4	< .001	12.4511***	0.3453	< .001	8.7733***	0.2964	< .001	8.3333***	0.3086
Time	2	0.220	1.0533NS	0.2674	< .001	5.3733***	0.2296	< .001	4.9586***	0.2302
Sterilant.Time	8	0.103	1.1644NS	0.598	0.547	0.44 NS	0.5133	0.589	0.4143NS	0.5206
Covariate								0.242	0.6992NS	
Residual	210		0.6902			0.5086			0.5077	
Total	224									
Summary of data										
s.e.			0.8308			0.7131			0.7125	
cv%			6.1			6.8			6.8	

*** Significant at $P<0.001$

NS Not significant

2.3.1 Effect of screened sterilants on shoot apex culture of *Acacia mearnsii*

The effect of sterilant on explant decontamination was significant. Use of 0.2% $HgCl_2$ was the most effective sterilant tested with regards to explant sterilisation, although it was not significantly different to 25% Jik ($p<0.05$) (Figure 2.2). Treatment with 7% $CaCl_2O_2$ was resulted in significantly lower decontamination of explants than any of the other sterilants tested ($p<0.05$) (Figure 2.2).

The effect of sterilant on shooting levels was found to be significant. Explants treated with 25% Jik showed significantly higher shooting levels than all the other sterilants tested ($p<0.05$) (Figure 2.2). $HgCl_2$ at 0.2% had the most damaging effect on shooting, although it was not significantly different to 7% $Ca(ClO)_2$ or 75% Jik ($p<0.05$).

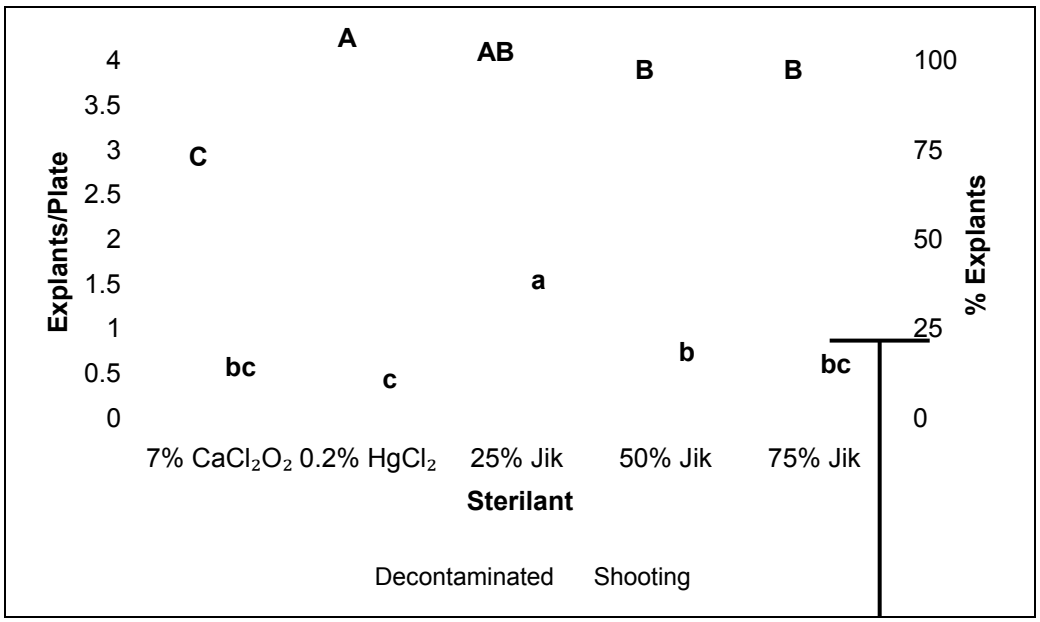

Figure 2.2 Mean explants, per plate, and the corresponding percentages of decontaminated and shooting explants after treatment with different sterilants. Treatments denoted with the same letters are not significantly different at the 0.05% level (upper and lower case letters refer to two separate data sets and can not be compared).

2.3.2 Effect of sterilant exposure time on shoot apex culture of *Acacia mearnsii*

The effect of sterilant exposure time on explant decontamination levels was not significant, however the effect of sterilant exposure time on shooting was found to be significant. Explants exposed to sterilants for 10 min showed significantly better shooting than other exposure times tested ($p<0.05$) (Figure 2.3). Explants exposed for 30 min demonstrated the lowest shoot production. However, these levels were not significantly different to the 20 min treatment ($p<0.05$).

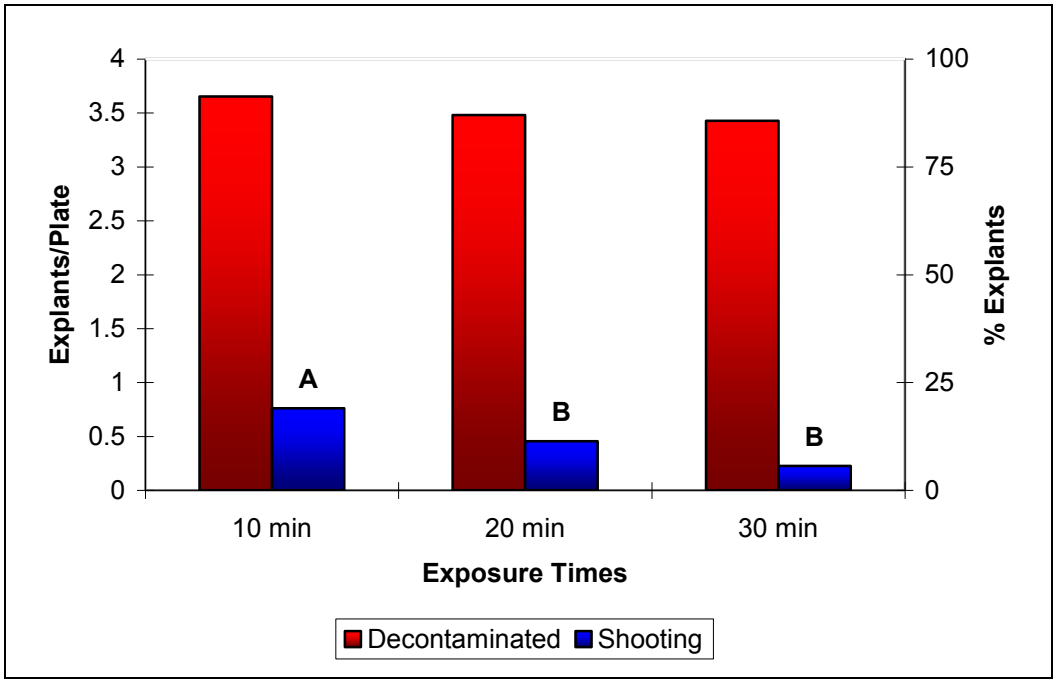

Figure 2.3 Mean explants, per plate, and the corresponding percentages of decontaminated and shooting explants after exposure to sterilants for different time periods. Treatments denoted with the same letters are not significantly different at the 0.05% level (only the shooting levels data set showed any significant differences)

2.3.3 Effect of sterilant by exposure time on shoot apex culture of *Acacia mearnsii*

The interaction effect of sterilant by exposure time was not significant on either decontaminated explant levels or shooting levels (Table 2.2).

2.3.4 Regression Analysis

A significant but weak positive linear correlation between shooting levels and decontaminated explant levels occurred, but this relationship accounted for only 1.8% of the total variance (Table 2.3).

Table 2.3 Summary of simple linear regression of transformed shooting data and transformed decontaminated explant data.

Source of variation	d.f.	s.s.	m.s.	v.r.	*F*. prob
Regression	1	3.5	3.4943	5.10	0.025
Residual	223	152.7	0.6846		
Total	224	156.2	0.6971		
r^2		1.8			
Std. Error		0.827			
Estimate of parameters					
		Estimate	s.e.	*t*(223)	*t* pr.
Constant		8.720	0.781	11.16	<.001
Transformed decontaminated data		0.1302	0.0576	2.26	0.025

A significant but weak negative linear correlation between shooting levels and exposure times and this relationship was found to account for only 6.4% of the total variance (Table 2.4).

Table 2.4 Summary of simple linear regression of transformed shooting data and exposure times.

Source of variation	d.f.	s.s.	m.s.	v.r.	*F.* prob
Regression	1	10.73.5	10.6667	16.35	<.001
Residual	223	145.5	0.6524		
Total	224	156.2	0.6971		
r^2		6.4			
Std. Error		0.808			
Estimate of parameters					
		Estimate	s.e.	*t*(223)	*t* pr.
Constant		11.013	0.142	77.30	<.001
Exposure time		-0.02667	0.00660	-4.04	<.001

2.4 DISCUSSION

The results show that sterilant and exposure time both influence the response of *A. mearnsii* shoot apexes to direct organogenesis. Although these factors show strong effects on their own, there seems to be no interaction between these two factors.

The effect of sterilant on explant decontamination levels was significant, with 0.2% $HgCl_2$ showing the lowest levels of contamination. This result supports earlier findings indicating that $HgCl_2$ was a suitable sterilant for tissue culture of *Acacia mearnsii* (Beck, 1999). However, it is important to note that Jik, a household bleach, at a concentration of 25%, produced decontamination levels similar to $HgCl_2$ (Figure 2.2). In contrast, 7% $CaCl_2O_2$ had significantly had significantly higher contamination levels than any of the other sterilants tested (Figure 2.2).

A significant positive linear correlation between shooting levels and decontaminated explant levels occurred. This relationship, however, accounted for only 1.8% of the total variance and, as to be expected from such a weak relationship, subsequent analysis of

shooting counts with decontaminated counts run as a covariate showed this covariate effect not to be significant.

The effect of sterilant on shooting levels was significant and explants treated with 25% Jik showed significantly higher shooting levels than all the other sterilants tested, including 0.2% $HgCl_2$, which had the most damaging effect on the explants and thus on shoot production (Figure 2.2). Although 0.2% $HgCl_2$ showed the lowest of shooting levels, this was not significantly different to 7% $CaCl_2O_2$ or 75% Jik (Figure 2.2). This result indicates, not only that 25% Jik is a viable surface sterilant alternative to 0.2% $HgCl_2$, but that it exhibits a less harmful effect on the shoot apex culture of *A. mearnsii*. This result is consistent with other findings that indicate $HgCl_2$ is detrimental to plant growth and that in some cases NaOCl (the active ingredient in Jik), has been reported to promote seedling growth (Chun *et al.*, 1997; Mor *et al.*, 2002).

The results indicate that, within the range tested, sterilant exposure time has no effect on decontamination levels, whilst the effect of sterilant exposure time on shooting was significant. These results are in direct conflict with previous studies that suggest exposure time plays a significant role in the degree of sterilisation (Beck, 1999). A significant negative linear correlation between shooting levels and exposure times suggests that the longer the explant is exposed to the sterilant, the greater the damage that is incurred. However, this relationship was found to be weak, accounting for only 6.4% of the total variance. Explants exposed to sterilants for 10 min showed significantly better shooting levels than other exposure times tested (Figure 2.3), whilst explants exposed for 30 minutes showed the greatest reduction in shoot production, although not significantly different to the 20 minute treatment (Figure 2.3).

Further examination of the results suggests that 25% Jik is a preferable alternative to other sterilants investigated. Furthermore Jik is handled in a safer manner than $HgCl_2$ and at low concentrations; it seems to cause lower phytotoxic effects. Results suggest that the detrimental effect of exposure time on the health of the explant far outweighs the perceived sterilisation benefit. Thus, from those treatments tested, Jik (NaOCl), at a

concentration of 25% (0.875%) and an exposure time of 10 min, best fulfils the requirements for a suitable sterilant of *A. mearnsii* shoot apex explants.

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CHAPTER 3

EFFECT OF HARVESTING SEASON AND MOTHER PLANT ON SHOOT APEX CULTURE OF *ACACIA MEARNSII*

ABSTRACT

The success of any tissue culture technique depends on a large array of *ex vitro* and *in vitro* variables. The objective of this research was to determine the effect of two *ex vitro* variables, season and mother plant, on shoot apex culture of *A. mearnsii*. Explants from four individual mother plants were cultured on MS medium supplemented with 2.0 mg L^{-1} BA during four separate seasons and monitored for 21 d for signs of contamination and shooting. Spring was the best harvesting season as spring-harvested explants showed significantly higher explant decontamination and shooting levels than explants harvested in the three other seasons. The effect of mother plant (genotype) on the performance of *A. mearnsii* explants during shoot apex culture was also found to be significant, especially with regard to shooting levels.

3.1 INTRODUCTION

Black wattle, *Acacia mearnsii* (de Wild.), was introduced to South Africa from Australia in 1864 and these early plantings were used to supply firewood, shelterbelts and shade for livestock (Sherry, 1971). Today black wattle is valued as a source of tannin, fuel, mine timber, parquet blocks, hardboard and rayon and in recent times it has become popular as a source of high quality pulp (Sherry, 1971; Jarmain and Lloyd-Jones, 1982; Beck, 1999; Dunlop and MacLennan, 2002). Black wattle's market flexibility, together with its good growth and wood properties, make it a commercially important species within the South African forestry industry (Dunlop *et al.*, 2000).

A phenotypic trait is made up of the sum of two major components, the genetic and environmental effects (Wricke and Eberhard Weber, 1986). Osborn observed variation of phenotypic traits amongst black wattle trees that he had planted in 1927. He noted that some individuals showed good vigour, form and resistance to disease (Dunlop and MacLennan, 2002). This phenotypic variation may extend to black wattle's responses to tissue culture and although reported in other species, it has not been formally reported for *Acacia mearnsii* (Banerjee *et al.*, 2007).

Tissue culture techniques have been attempted on a large range of tree species. The success of such attempts depends on the complex interactions between explant, plant growth regulators, culture conditions and genotype (Nehra and Kartha, 1994). Explant material grown *ex vitro* is exposed to a countless array of possible tissue culture contaminants and microbes and certain climatic conditions (usually higher temperatures) promote bacterial and fungal growth (Etchells *et al.*, 1973). The abundance of fungi and bacteria in the environment has been shown to vary according to season (Matheron *et al.*, 2005; Ipsilantis and Sylvia, 2007). It therefore seems likely that the harvest season may have an important effect on the response of *A. mearnsii* to shoot apex culture. This may be due to environmental factors influencing the growth of infecting microbes or the health and physiological status of the plant itself, or more likely, a combination of both (Siril and Dhar, 1997; Bhatt and Dhar, 2004).

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Four maternally unrelated two-year-old black wattle trees growing in 50 L black cultivation bags were utilised to provide explants. The maternal history of these trees was known, but for simplicity, they are referred to here as individuals A1, A2, A3 and A4. The trees were kept under 20% shade cloth at the Institute for Commercial Forestry Research (ICFR) nursery and were watered regularly via an automated irrigation system to prevent the bags from drying out. Explants were harvested from the trees during March, June, September and December of 2004.

3.2.2 Selection and collection of shoot apexes

Healthy young shoots comprising of up to six shoot apexes were selected, resulting in a greater chance of mitotic division and thereby increasing the chance of regeneration during micro-propagation. These shoots were carefully removed, during the mid-morning, from a particular tree by hand and placed in a sterile petri dish, labelled with the corresponding tree identity. From each tree 40 shoot apexes were collected and up to 4 individual trees were harvested on each collection day. Shoots were harvested once every 3 months and as close to the middle of each season as possible.

3.2.3 Topical sterilization

The harvested shoots were taken in sterile petri dishes directly from the nursery to the ICFR laboratory where they were exposed to a topical sterilisation process to remove any surface contaminants. All collected shoots underwent the following topical sterilisation process:

Step 1 Thoroughly wash under running tap water
Step 2 Surface sterilise in 70% ethanol for 1 min
Step 3 Soak in 0.2% Benlate® (benomyl 50% a.i.; Du Pont de Nemours, South Africa) for 10 min
Step 4 Rinse in distilled water
Step 5 Soak in 25% Jik (0.875% $NaOCl_2$) for 10 min
Step 6 Rinsed in distilled water
Step 7 Soaked in 500 mg l^{-1} ascorbic acid for 15 min

3.2.4 Dissection, culture media and growth conditions

Isolation of the shoot apexes through the dissection of the shoots was undertaken on a laminar flow bench with a sterile scalpel. The shoot apexes were then carefully placed on culture media using sterile tweezers.

Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} BA was chosen as the shoot-initiation medium as it was shown to be successful with *A. mearnsii* micropropagation in the past (Murashige and Skoog, 1962; Beck *et al.*, 1998; Beck,

1999; Beck *et al.*, 2000). The medium was supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} myo-inositol, altered to a pH of 5.8. Gelrite 3 g L^{-1} (solidifying agent) was added prior to the medium being autoclaved (at 121°C and 103 kPa for 20 min). Ten plates (65 mm sterile plastic petri-dishes from Labotec), each containing four shoot apexes, were used for each treatment and sealed using parafilm. All cultures were maintained at a temperature of 25 ± 2°C, incubated under continuous light (23 µmol m^{-2} s^{-1}) with a photoperiod of 16 h light / 8 h dark. Cool white fluorescent tubes provided the light source. Recipes of all basal media tested are listed in Appendix 1 and all culture protocols were carried out using aseptic technique.

3.2.5 Monitoring and quantification of results

In order to quantify the effect of season and mother plant individual on shoot apex culture, it was necessary to place explants on to the culture medium and initiate shoots. Treatments were screened not only for contamination levels, but also for the most successful shooting rate. The apexes were monitored on a weekly basis for 21 d. On each occasion an explant became contaminated, the surviving explants were transferred to a new plate. Three weeks after initial placement on culture medium the number of decontaminated explants (free of visible microbial growth), together with the number of shooting explants in each plate was recorded. Shooting was defined by a visual doubling in length of the explant, excluding callus growth.

3.2.6 Statistical analysis

Statistical analysis was carried out using Genstat Version 9.1. Data was transformed according to the following linear transformation: $y = x + 10$; where x is the original data and y the transformed data. A two-way (season and individual) ANOVA without blocking was performed on the transformed data and the results were recorded. This analysis was performed on both data sets. A linear regression was performed between transformed shooting data and transformed decontaminated explant data.

3.3 RESULTS

Results are shown without transformations, unless specified, as the linear transformation did not alter significance levels, only the co-efficient of variation. The results of both the two-way ANOVA analyses (decontaminated- and shooting data) showed differing levels of significance (Table 3.1). Subsequent analysis of shooting counts with decontaminated counts run as a covariate showed this covariate effect to be significant (Table 3.1). Therefore, some of the shooting results, where specified, have been shown adjusted for the decontaminated covariate.

Table 3.1 Summary of the analyses of variance for decontaminated-, shooting- and shooting counts adjusted for the covariate, decontaminated counts. All data was transformed according a linear transformation prior to analysis and least significant differences of means (l.s.d.) are listed at the 5% level.

	Decontaminated			**Shooting**			**Shooting with covariate**		
Source of variation									
	d.f.	m.s.	l.s.d.	d.f.	m.s.	l.s.d.	d.f.	m.s.	l.s.d.
Date	3	93.8167***	0.4138	3	64.6229***	0.3221	3	2.1101***	0.3201
Individual	3	2.7833*	0.4138	3	10.8729***	0.3221	3	11.9272***	0.2451
Date.Indi.	9	3.5444***	0.8275	9	7.2507***	0.6443	9	3.413***	0.5245
Covariate							1	33.4786***	
Residual	144	0.8764		144	0.5313		143	0.3008	
Total	159			159			159		
Summary of data									
s.e.	0.9362			0.7289			0.5485		
cv%	8.1			6.6			5		

*** Significant at $P<0.001$

* Significant at $P<0.05$

3.3.1 Climatic conditions for Pietermaritzburg

Pietermaritzburg is a typically summer rainfall area (Figure 3.1). Summer temperature and rainfall figures are high and become lower during the winter months.

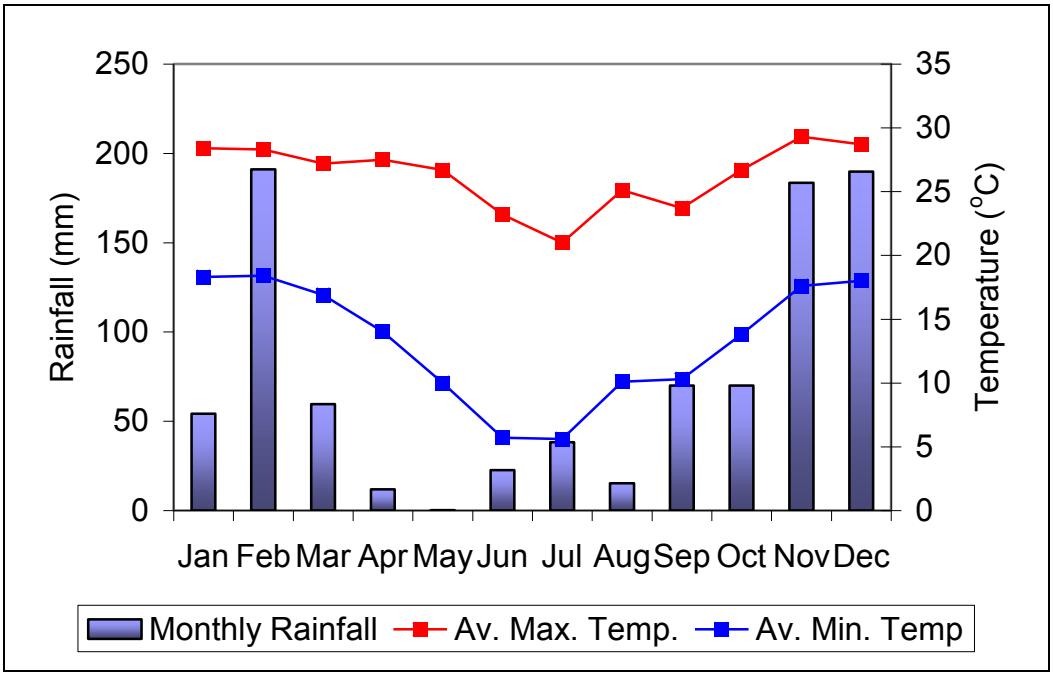

Figure 3.1 Climatic data for Pietermaritzburg, South Africa, during 2004. Monthly rainfall and average maximum and minimum temperatures are shown. Data provided by the South African Weather Bureau, station number 02396985.

3.3.2 Effect of harvesting season on shoot apex culture of *Acacia mearnsii*

The effect of harvest season on decontaminated explant levels was significant. September (spring) showed the highest level of explant decontamination explant levels, significantly better than that of all other seasons tested ($p<0.05$) (Figure 3.2). December (summer) showed the highest levels of contaminated explant levels, which were significantly worse than ant of the other seasons tested ($p<0.05$) (Figure 3.2).

The effect of harvest season on shooting levels was significant. Explants harvested in September showed significantly higher shooting levels than all the other seasons tested ($p<0.05$) (Figure 3.2). December showed the least shooting, although not significantly less than March ($p<0.05$). When this analysis was performed with decontaminated data as a covariate, there were no significant ranking changes at the 0.05% level. Thus results are shown without any covariance adjustments (Figure 3.2)

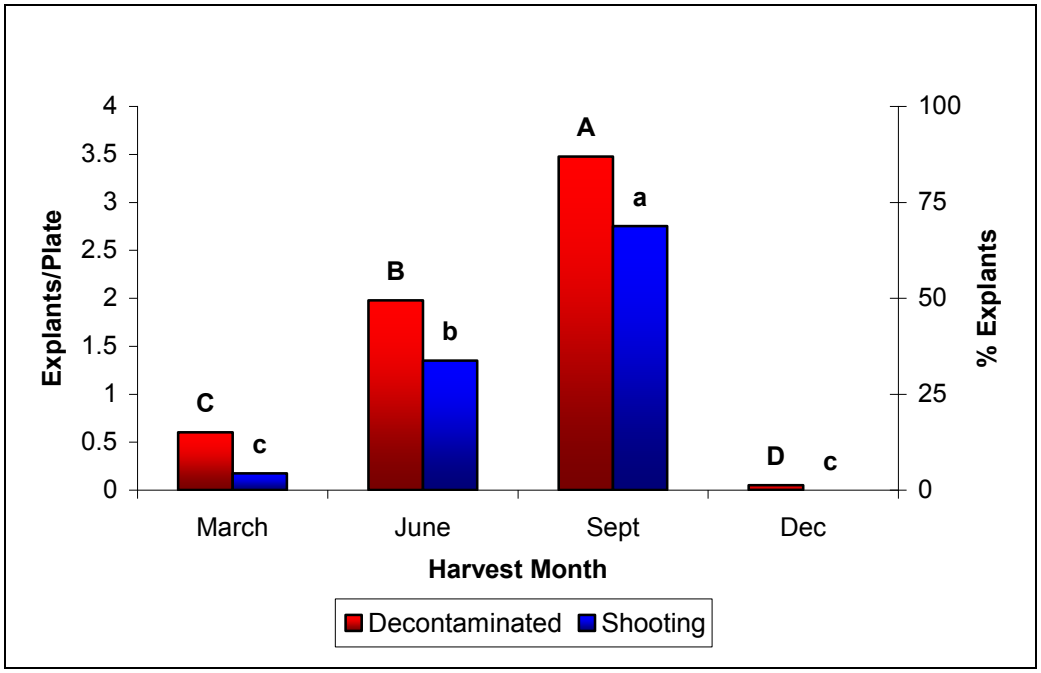

Figure 3.2 Mean explants, per plate, and the corresponding percentages of decontaminated and shooting explants harvested during different months. Treatments denoted with the same letters are not significantly different at the 0.05% level (upper and lower case letters refer to two separate data sets and cannot be compared).

3.3.3 Effect of individual mother plant on shoot apex culture of *Acacia mearnsii*

The effect of individual mother plant on decontaminated explant levels was significant. Individual A2 showed the highest decontamination, although not significantly better than individual A4 ($p<0.05$) (Figure 3.3). Individual A3 showed the worst decontamination, although not significantly lower than individual A1 ($p<0.05$).

The effect of individual mother plant on shooting levels was significant. Explants harvested from individual A2 showed significantly higher shooting levels than all the other individuals tested ($p<0.05$) (Figure 3.3). Individual A4 showed the least shooting significantly less than all other individuals tested ($p<0.05$). When this analysis was performed with decontaminated data as a covariate, there were no

changes to the shooting ranking and only a single change to significance groupings. Thus results are shown without any covariance adjustments (Figure 3.3)

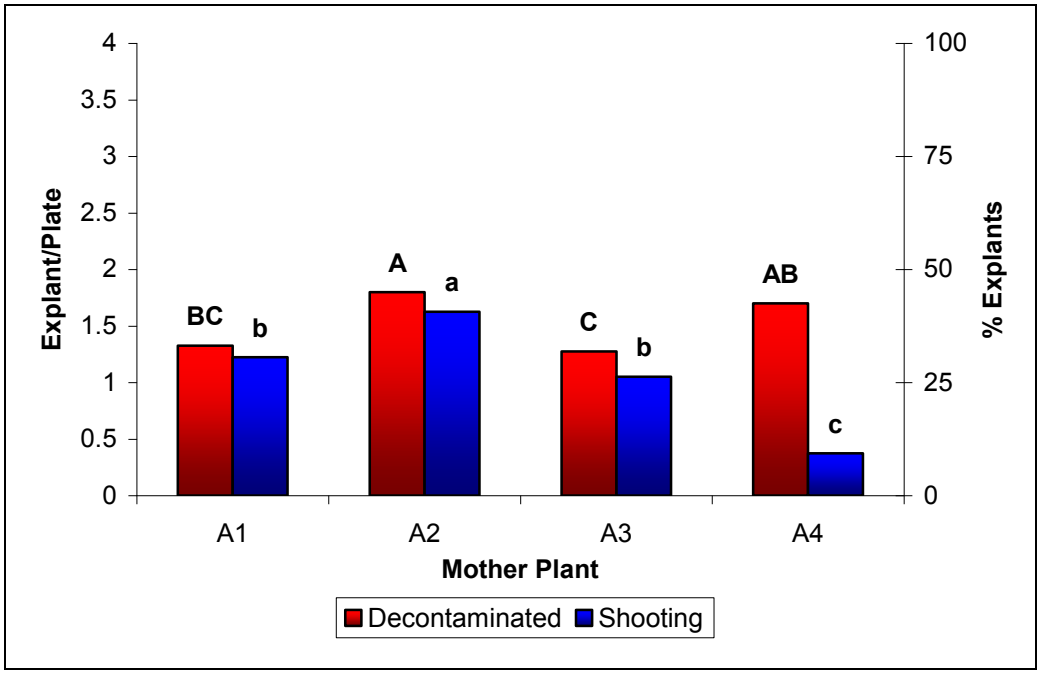

Figure 3.3 Mean explants, per plate, and the corresponding percentages of decontaminated- and shooting explants harvested from different mother plants. Treatments denoted with the same letters are not significantly different at the 0.05% level (upper and lower case letters refer to two separate data sets and can not be compared).

3.3.4 Effect of harvest season by individual on shoot apex culture of *Acacia mearnsii*

The interaction effect of harvest season by individual on decontaminated explant levels was significant. The treatments showing the best decontamination rates were harvested in September, although not significantly different from some treatments harvested in June ($p<0.05$) (Figure 3.4). The treatments showing the lowest decontaminated rates were all harvested in December. Three of the treatments harvested in December resulted in complete contamination, without even one explant surviving, but this result was not significantly different from three treatments harvested in March and one harvested in June ($p<0.05$) (Figure 3.4).

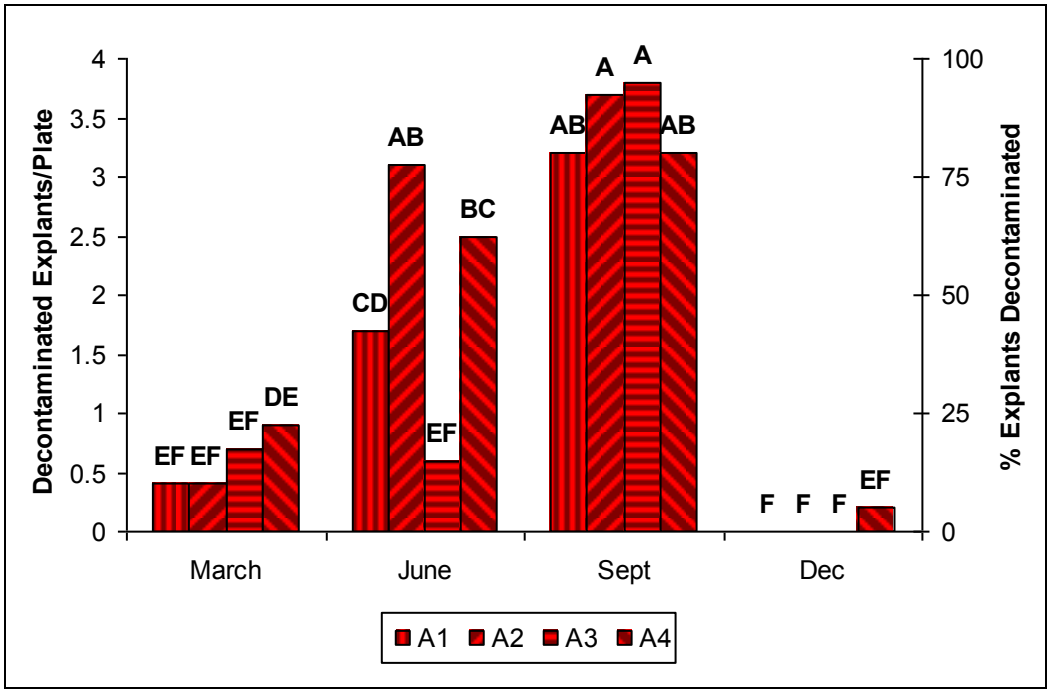

Figure 3.4 Mean explants, per plate, and the corresponding percentages of decontaminated explants harvested from different mother plants at different times of the year. Treatments denoted with the same letters are not significantly different at the 0.05% level.

The interaction effect of harvest season by individual on shooting levels was found to be significant. The three best treatments with regards to shooting levels were all harvested in September, however these were not significantly different to one of the treatments harvested in June ($p<0.05$) (Figure 3.5). The rankings and significance levels change extensively when they were adjusted to incorporate decontaminated data as a covariate, therefore results are also shown adjusted for the covariate (Figure 3.6).

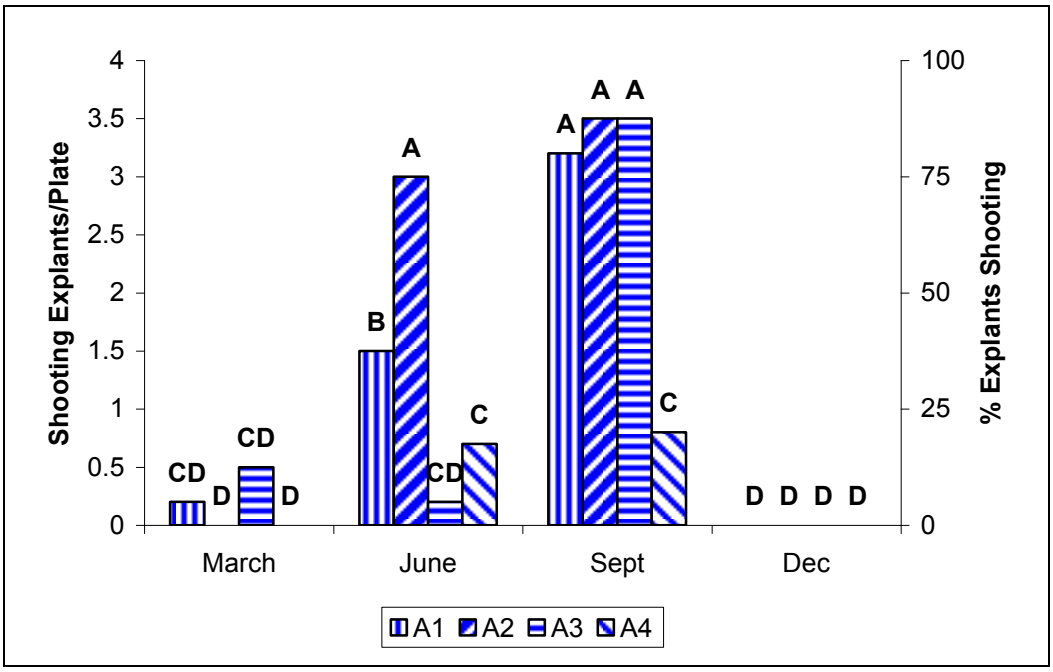

Figure 3.5 Mean explants, per plate, and the corresponding percentages of shooting explants harvested from different mother plants at different times of the year. Treatments denoted with the same letters are not significantly different at the 0.05% level.

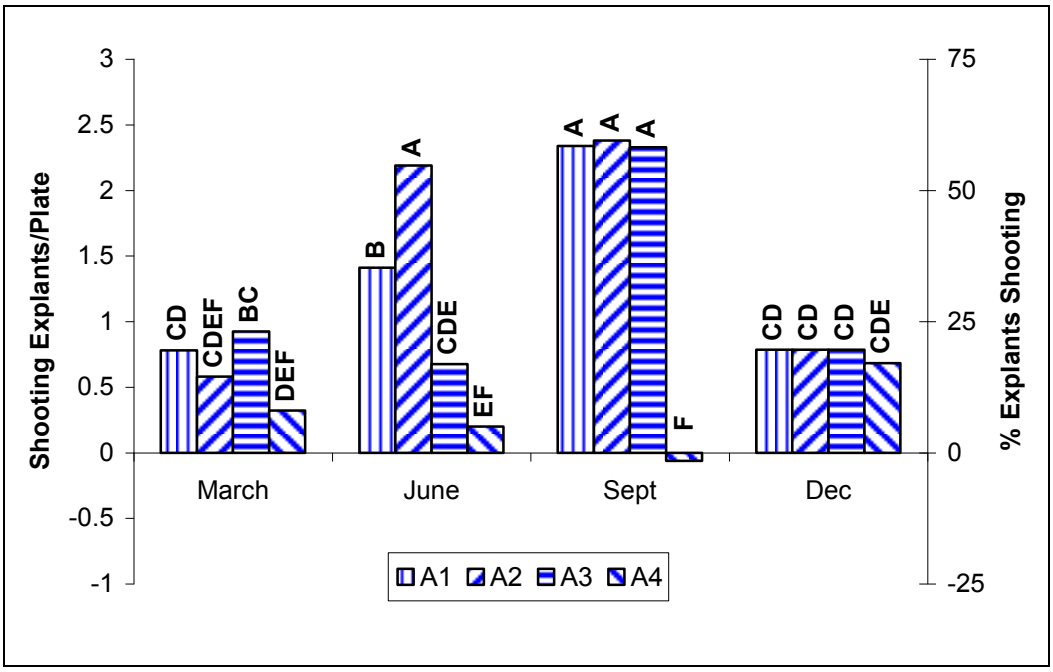

Figure 3.6 Mean explants, per plate, and the corresponding percentages of shooting explants harvested from different mother plants at different times of the year. Results are shown adjusted for the covariate (decontaminated) and treatments denoted with the same letters are not significantly different at the 0.05% level.

3.3.5 Regression Analysis

A significant positive linear correlation between shooting levels and decontaminated explant levels occurred and this relationship accounted for 68.3% of the total variance (Table 3.2).

Table 3.2 Summary of simple linear regression of transformed shooting data and transformed decontaminated explant data.

Source of variation	d.f	s.s.	m.s.	v.r.	*F*. prob
Regression	1	252.4	252.394	344.22	<.001
Residual	158	115.8	0.7332		
Total	159	368.2	2.316		
r^2		68.3			
Std. Error		0.856			
Estimate of parameters					
		Estimate	s.e.	*t*(158)	*t* pr.
Constant		2.417	0.471	5.13	<.001
Transformed decontaminated data		0.7507	0.0405	18.55	<.001

3.3 DISCUSSION

The results show that both harvest season and individual mother plant both play a role in the response *Acacia mearnsii* has to shoot apex culture. These two factors not only show strong effects on their own, but also exhibit a strong interaction effect between these two factors.

A significant positive linear correlation occurred between decontaminated explant levels and shooting levels. This result is not surprising when dealing with low levels of decontaminated explants, because contaminated explants are out-competed and do not form shoots. Therefore shoots can only form from decontaminated explants and are directly dependant on this variable. This relationship accounted for 68.3% of the total variance and, thus, when subsequent analysis was performed with decontaminated data run as a covariate, the covariate effect accounted for a large proportion of the total variance (Table 3.1). This result contrasts the results of the previous chapter where decontaminated levels did not affect shooting levels significantly (Table 2.2). This suggests that it is difficult to compare shooting levels when decontaminated explant levels vary greatly, and are much more reliable when decontaminated levels vary by a smaller degree and remain above 50%.

The effect of harvest season on decontaminated explant levels was significant. September (spring) showed the highest decontaminated levels, significantly higher than any other harvest season tested (Figure 3.2). Alternatively December (summer) showed lower decontaminated explant levels than any other harvest season results (Figure 3.2). This result suggests that during spring, the condition of the mother plants are physiologically more suitable than during other seasons and as such, the plants host defences are better equipped to combat pathogens in spring. This observation is consistent with other work done on the susceptibility of *Gleditsia triacanthos* seedlings to fungus inoculation during different seasons (Jacobi, 1992). However, this must be viewed in a geographical climatic context and may vary at a species level, as indicated in other micropropagation studies using *Myrica esculenta*, where winter was shown to be the most suitable season for explant harvests (Bhatt and Dhar, 2004).

The effect of harvest season on shooting levels was also significant. Re-analysing this with decontaminated data as a covariate greatly reduced the amount of variance attributed to by season, but it did not affect the rankings or the significance groupings. Explants harvested in September showed the highest shooting levels, significantly higher than any other harvest season tested. Explants harvested in December showed the lowest shooting levels, although not significantly lower than those harvested in March (autumn). These results confirmed that spring is the optimal season to harvest shoot apex explants for *Acacia mearnsii* micropropagation.

The effect of the individual mother plant on decontaminated explant levels was found to be significant. Individual A2 showed the highest decontaminated levels, but was not significantly different to individual A4. Alternatively, A3 showed the lowest decontaminated explant levels, but this result was not significantly different to individual A1. The variability in individual mother plant may indicate a genotype effect on decontaminated levels, although it is more likely that this result is due to the slight differences in the bagged mother plants environment. Clarification of this genotype by environment effect would necessitate testing of numerous cloned individuals and falls outside the scope of this investigation.

The results show the effect of individual mother plants on explant shooting levels to be significant. Individual A2 exhibited significantly higher decontaminated explant levels than any other mother plants tested, whilst A4 showed significantly lower levels than the others. Subsequent analysis performed with decontaminated data as a covariate had no effect on individual rankings; however A1 was no longer significantly worse than A2. Whilst differences in shooting levels were strongly influenced by decontaminated levels, it is important to note that individual A4 showed good decontaminated explant levels, yet explants derived from this individual resulted in the lowest shooting levels. This result indicates that shooting may be influenced by the genotype of the mother plant, because it seems unlikely that environment would negatively influence shooting without showing a similar effect on decontaminated levels. This hypothesis, although reported in other species, requires further validation for *Acacia mearnsii* (Banerjee *et al.*, 2007).

The interaction effect of season by individual had a significant influence on decontaminated explant levels. With the exception of individual A3 harvested in June (winter), which showed significantly lower decontaminated explant levels than other individuals harvested during this season, results followed the aforementioned seasonal trend. Whilst the interaction effect of season by individual on shooting was also significant, these results are less reliable due to the strong effect decontaminated data has on them. This is especially true of treatments that show low decontaminated explant levels (Figure 3.4) and is illustrated by comparing data analysed without decontaminated data as a covariate (Figure 3.5) and data analysed with this covariate (Figure 3.6). Data with very low decontaminated explant levels (below 25%), show the biggest changes and prove the most unreliable, due to the small sample size.

Results suggest that individual mother plants have an important effect on shoot apex culture of *Acacia mearnsii*. Whilst the effect of individual on sterilisation can easily be attributed to slight differences in bagged tree environment, the poor shooting performance of individual A4, when examined with its high decontaminated explant levels, seems to indicate a genotypic effect. Further examination of the results indicates that the best time for explant harvesting, regardless of mother plant, is during late winter and spring. However, if this result is to be useful, it must be viewed

in the context of the local weather patterns, because climate may affect the response *Acacia mearnsii* has to shoot apex culture (Figure 3.1). This may be due to environmental factors influencing the growth of infecting microbes or the health of the plant itself, or, more likely, a combination of both (Siril and Dhar, 1997; Bhatt and Dhar, 2004). In other wintergreen species the importance of old leaves survival for phenological events that lead to the mobilisation of resources toward the shoot apical meristem in early spring has been studied and it is probable that a similar mobilisation of resources in *Acacia mearnsii* positively influences its shoot apex culture during spring (Damascos *et al.*, 2005).

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CHAPTER 4

EFFECT OF RED LIGHT AND AN ALTERNATIVE BASAL MEDIUM ON SHOOT ELONGATION OF *ACACIA MEARNSII* DURING SHOOT APEX CULTURE

ABSTRACT

Shoot elongation is an important morphological process in most tissue culture techniques. The objective of this study was to test an alternative basal medium and the effect of red light on the shoot elongation of *Acacia mearnsii* during shoot apex culture. Two independent experiments were undertaken, comparing shoot elongation on Woody Plant Medium (WPM) to the Murashige and Skoog (MS) basal medium control, and shoot elongation under a red cellophane box, compared to control culture light conditions. Although results were not significant, shoots under the red light showed a weak trend of increased elongation. The spectral irradiance under red cellophane was also tested and the results indicated this may be a viable alternative to expensive red light sources.

4.1 INTRODUCTION

Numerous techniques are being utilised for the micropropagation of forest trees. One such technique, meristem culture, makes use of the totipotent cells at the tip of the shoot apical meristem to produce contaminant-free plants with reduced levels of pathogens (Bonga and von Aderkas, 1992; Nehra and Kartha, 1994). The meristem region is very small and establishing successful cultures from such a small explant can be problematic (Bonga and von Aderkas, 1992). The shoot apex is the name given to the apical meristem, together with the upper layers of leaf primordia, which occur just below the apical dome (Dodds and Roberts, 1985). This shoot apex region, while more likely to contain pathogens than the meristem region alone, may be more suited to use in large scale tissue culture, because it makes use of a larger explant.

Although substantial research has been conducted on the tissue culture of *Acacia* species, resulting in varying degrees of plantlet regeneration, micropropagation research on *A. mearnsii* is still limited (Beck, 1999). Shoot elongation is an important step in the successful micropropagation of any plant and forms the important link between shoot initiation and root initiation (Beck *et al.*, 2000). Although shoot elongation of *A. mearnsii* has been achieved, it remains a problem when utilising small explants from older, *ex vitro* grown material (Huang *et al.*, 1994; Correia and Graça, 1995; Beck *et al.*, 2000).

The responses of explants in tissue culture are affected by the composition of the supporting medium, the temperature of incubation, the wavelength, intensity and period of lighting conditions and the nutrient and growth hormone levels (Beck, 1999). These factors, both physical and chemical, combine in complex interactions and may have positive or negative effects on the growing explants. Most *A. mearnsii* micropropagation studies utilise MS medium as a basal medium (Murashige and Skoog, 1962; Huang *et al.*, 1994; Correia and Graça, 1995; Beck *et al.*, 1998; Quoirin *et al.*, 1998; Beck, 1999; Quoirin *et al.*, 2001; Quoirin *et al.*, 2004). Although there has been some success using WPM, this medium has not been utilised extensively with regard to *A. mearnsii* (Lloyd and McCowan, 1981; Beck *et al.*, 2000). In the past, inducing shoot elongation during micropropagation of *A. mearnsii* has been attained through the addition of plant growth regulators and other supplements to the basal culture medium (Huang *et al.*, 1994; Correia and Graça, 1995; Quoirin *et al.*, 1998; Beck, 1999; Quoirin *et al.*, 2001). Some micropropagation methods in other species have utilised red light as a means of promoting shoot elongation and although this manipulation of a light source is often costly, it can be very effective (Appelgren, 1991; Nhut *et al.*, 2003). This is because phytochrome, one of the two main light quality detecting pigments in plants, is most sensitive to red and far-red light (Moreira da Silva and Debergh, 1997). In this study the effects of an alternative basal media, WPM, and an affordable source of red light were individually evaluated for their effects on the shoot elongation of *A. mearnsii* during shoot apex culture.

4.2 MATERIALS AND METHODS

This study consisted of two separate elongation investigations. In order to carry out investigations on shoot elongation, it was first necessary to regenerate a large number of shoots. Since each plated explant does not necessarily develop into a regenerated shoot, a large number of shoot apexes needed to be cultured in order to produce enough regenerated shoots to study shoot elongation.

4.2.1 Plant material

Twelve two-year-old black wattle trees growing in 50 L black cultivation bags were utilised to provide explants. Only the maternal histories of these trees were known, making it impossible to know whether the trees were unrelated, half-sibs or full sibs, thus for simplicity the trees were assigned individual codes B1 to B12. The trees were kept under 20% shade cloth at the Institute for Commercial Forestry Research (ICFR) nursery and were watered regularly via an automated irrigation system to prevent the bags from drying out. Explants were harvested from the trees during 2004.

4.2.2 Selection and collection of shoot apexes

Healthy young shoots comprising of up to six shoot apexes were selected, in order to increase the chances of mitotic division and thereby increasing the chances of regeneration during micro-propagation. These shoots were carefully removed by hand, during the mid-morning, from a particular tree and placed in a sterile petri dish, labelled with the corresponding tree identity. From each tree 40 shoot apexes were collected and up to 4 individual trees were harvested each collection day. Each tree was given a recovery time of at least two months between harvesting.

4.2.3 Topical sterilization

The harvested shoots were placed in sterile petri dishes and taken directly from the nursery to the ICFR laboratory where they were exposed to a topical sterilisation

process to remove any surface contaminants. All collected shoots underwent the following topical sterilisation process:

Step 1 Thoroughly wash under running tap water
Step 2 Surface sterilise in 70% ethanol for 1 min
Step 3 Soak in 0.2% Benlate® (benomyl 50% a.i.; Du Pont de Nemours, South Africa) for 10 min
Step 4 Rinse in distilled water
Step 5 Soak in 25% Jik (0.875% $NaOCl_2$) for 10 min
Step 6 Rinsed in distilled water
Step 7 Soaked in 500 mg L^{-1} ascorbic acid for 15 min

4.2.4 Dissection and shoot-initiation

Isolation of the shoot apexes through the dissection of the shoots was conducted on a laminar flow bench with a sterile scalpel. The shoot apexes were then carefully placed on culture media using sterile tweezers.

Murashige and Skoog medium, supplemented with 2.0 mg L^{-1} BA, was chosen as the shoot-initiation medium because it was shown to be successful with *A. mearnsii* micropropagation in the past (Murashige and Skoog, 1962; Beck *et al.*, 1998; Beck, 1999; Beck *et al.*, 2000). The medium was supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} myo-inositol, altered to a pH of 5.8, and 3 g L^{-1} of Gelrite (solidifying agent) which was added prior to the medium being autoclaved (at 121°C and 103kPa for 20 min). Each plate (65 mm sterile plastic petri-dishes from Labotec) contained four shoot apexes from the same mother plant and was sealed using parafilm. All cultures were maintained at a temperature of 25 ± 2°C, incubated under continuous light (23 µmol m^{-2} s^{-1}) with a photoperiod of 16 h light / 8 h dark. Cool white fluorescent tubes provided the light source. Recipes of al nutrient media testes are reported in Appendix 1 and all culture protocols followed aseptic techniques.

The apexes were monitored on a weekly basis until a suitable number of initiated shoots were available for further investigation. When an explant became contaminated, the surviving explants were transferred to a new plate. Shooting was defined by a visual doubling in length of the explant, excluding callus growth. After sufficient numbers of shoots had been induced, callus tissue was carefully and

aseptically excised and the remaining shoot was transferred onto a shoot elongation medium.

4.2.5 Basal media investeigation

Woody Plant Medium (WPM) (Lloyd and McCowan, 1981) was investigated as a means of encouraging shoot elongation of *A. mearnsii* during shoot apex culture. WPM was compared to a MS medium control. Both media were supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} Myo-inositol, altered to a pH of 5.8. Gelrite 3 g L^{-1} (solidifying agent) was added prior to the media being autoclaved (at 121°C and 103 kPa for 20 min). Ten sterile 125 ml glass bottles, half filled with culture medium and each containing 3 regenerated shoots (30 in all) were used for each treatment. The mother plant identity for each shoot was known and recorded by numbering each shoot position on the outside of each glass bottle. Treatments had identical mother plant proportions (Table 4.1).

Table 4.1 Summary of explant sources, showing individual mother plant compositions for both treatments investigated.

Bottle No.	Mother Plant Composition	
	MS	WPM
1	3 B2	3 B2
2	3 B2	3 B2
3	3 B2	3 B2
4	3 B2	3 B2
5	3 B2	3 B2
6	2 B2; 1 B5	2 B2; 1 B5
7	3 B6	3 B6
8	2 B6; 1 B3	2 B6; 1 B3
9	3 B7	3 B7
10	3 B4	3 B4

All cultures were maintained at a temperature of 25 ± 2°C, incubated under continuous light (23 µmol m^{-2} s^{-1}) with a photoperiod of 16 hours light / 8 hours dark. Cool white fluorescent tubes provided the light source and cultures were monitored

on a weekly basis for 14 d. Recipes of all nutrient solutions are listed in Appendix 1 and all culture protocols were performed using aseptic techniques.

4.2.6 Investigating red light

Red light was investigated as a means of encouraging shoot elongation of *Acacia mearnsii* during shoot apex culture. In order to provide a cheap and effective way of supplying the explants with red light, a wooden frame was designed and built. The frame was 100 mm high, 260mm wide and 260 mm long. Around this box, coloured red cellophane was attached and the explant bottles were then placed in the box and exposed to control culture conditions (Figure 4.1). The effect the red box had on the culture light conditions was tested using a portable spectroradiometer. This machine performed three scans for each nanometre between 300 nm and 1100 nm and recorded the mean. The effect of the red box was compared to the control in standard culture light from fluorescent tubes.

Figure 4.1 Coloured cellophane boxes placed in an incubator and housing explant bottles.

To determine the effect of red light on shoot elongation of *A. mearnsii*, ten 125 ml sterile glass bottles each containing 3 regenerated shoots (30 in all) were utilised for each treatment. The mother plant identity for each shoot was known and recorded by numbering each shoot position on the outside of each glass bottle. Treatments had identical mother plant proportions (Table 4.2).

Table 4.2 Summary of explant source, showing individual mother plant compositions for both treatments investigated.

Bottle No.	Mother Plant Composition	
	White	Red
1	3 B1	3 B1
2	3 B2	3 B2
3	3 B2	3 B2
4	3 B2	3 B2
5	3 B4	3 B4
6	2 B9; 1 B2	2 B9; 1 B2
7	2 B5; 1 B10	2 B5; 1 B10
8	2 B8; 1 B11	2 B8; 1 B11
9	3 B12	3 B12
10	3 B12	3 B12

All cultures were maintained at a temperature of 25 ± 2°C, incubated under continuous light (23 µmol m^{-2} s^{-1} for the control) with a photoperiod of 16 hours light / 8 hours dark. Cool white fluorescent tubes provided the light source and cultures were monitored on a weekly basis for 14 d. Both treatments utilised MS mediu supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} Myo-inositol, altered to a pH of 5.8. Gelrite 3 g L^{-1} (solidifying agent) was added prior to the medium being autoclaved (at 121°C and 103 kPa for 20 min).

4.2.7 Monitoring and quantification of results

In order to measure shoot elongation during the two week elongation phase, a sterile system of measuring shoot length was devised. A ruler showing millimetre detail was photocopied onto an overhead transparency and then thoroughly washed in 70%

ethanol and placed on the laminar flow bench. A sterile glass petri-dish was then placed on top of the ruler. Shoots were then transferred onto the petri-dish in order to conduct measurements (Figure 4.2). Each shoot was measured before the elongation phase and this measurement together with its position in the jar and its parent tree was noted. After 14 d the length of each shoot was again measured and this together with the condition of the explant was noted.

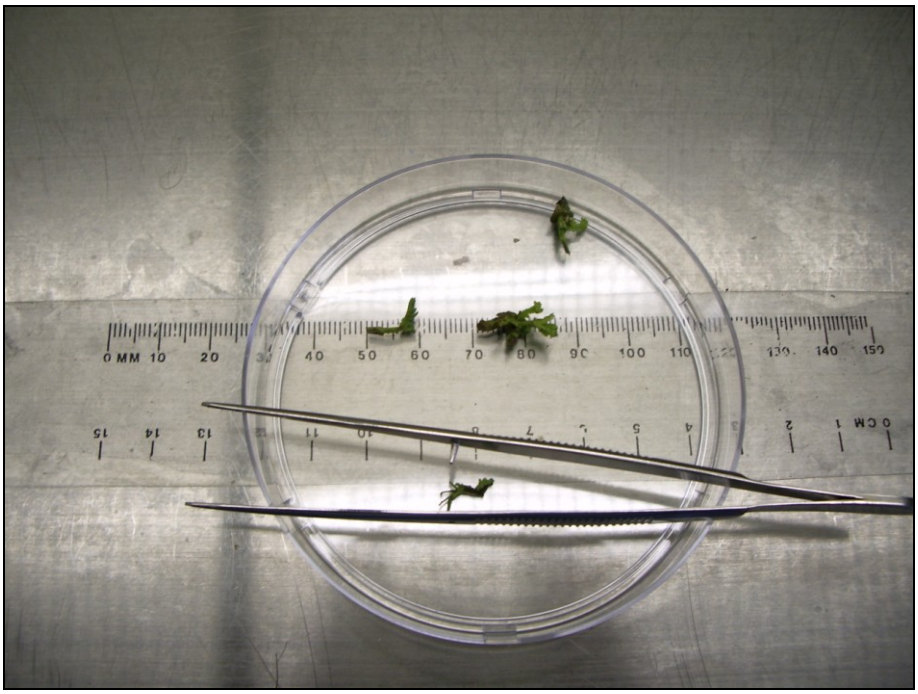

Figure 4.2 Measuring apparatus used to quantify results.

4.2.8 Statistical analysis

Statistical analysis was carried out using Genstat Version 9.1. The mean elongation of each plate was calculated and treated as one data point, as was each plate's survival count. A paired t-test was carried out on both pairs of data.

4.3 RESULTS

4.3.1 Effect of WPM as an alternative basal medium on shoot elongation

The effect of basal medium on shoot elongation was not significant. The probability of the null hypothesis (no difference in means) for both shoot elongation and shoot survival was high (Table 4.3). The difference between the two treatment means was small with no treatment trends observed (Figure 4.3).

Table 4.3 Summary of paired t-test performed on mean explant per plate elongation and on total survival per plate for the two basal media tested.

	Elongation		**Survival**	
	WPM	MS	WPM	MS
Mean	1.767	1.867	2.100	1.900
Variance	3.532	3.684	1.211	0.989
Hypothesized Mean Difference	0		0	
d.f.	9		9	
s.e.	0.5283		0.2906	
t Stat	-0.19		0.69	
P(T<=t)	0.854		0.509	

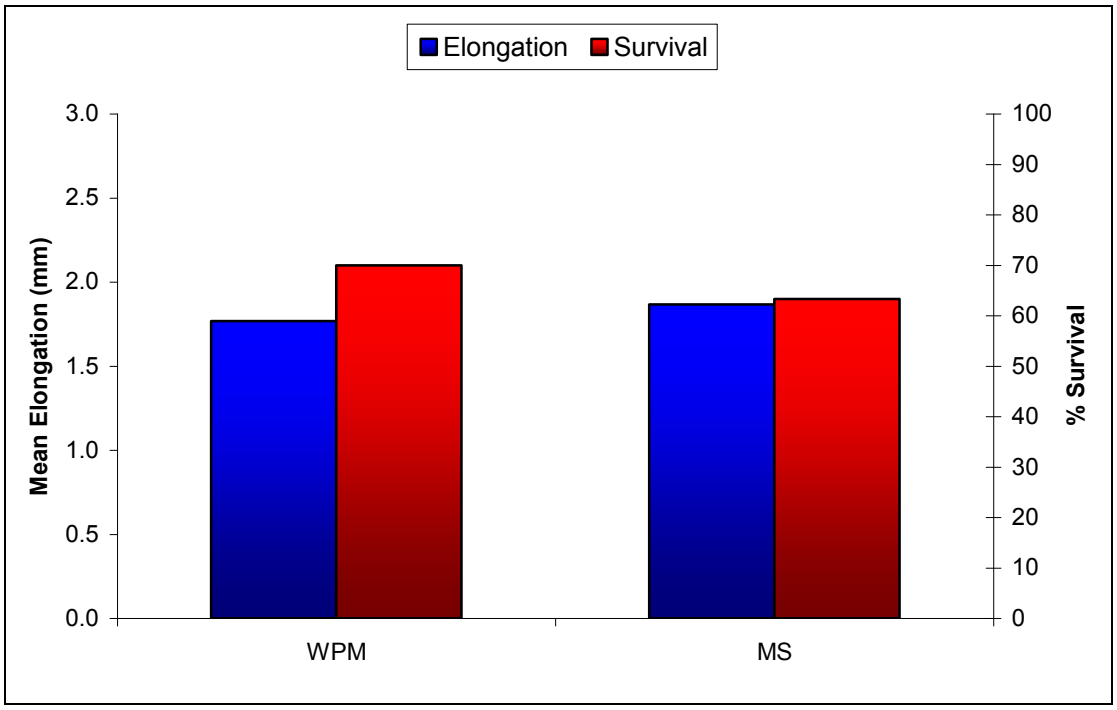

Figure 4.3 Mean explant elongation per plate and percentage survival per plate for the two basal media tested.

4.3.2 Effect of the red cellophane box on culture light conditions

The red cellophane box reduced the spectral irradiance in all parts of the visible spectrum, except the wavelengths associated with red light (Figure 4.4). The peak irradiance under the red box was situated at 605 nm, whilst that of the control was at 546 nm. The proportion of red light as compared to other wavelengths was greatly increased under the red box and as such, the effect of the cellophane box on the culture light was deemed satisfactory.

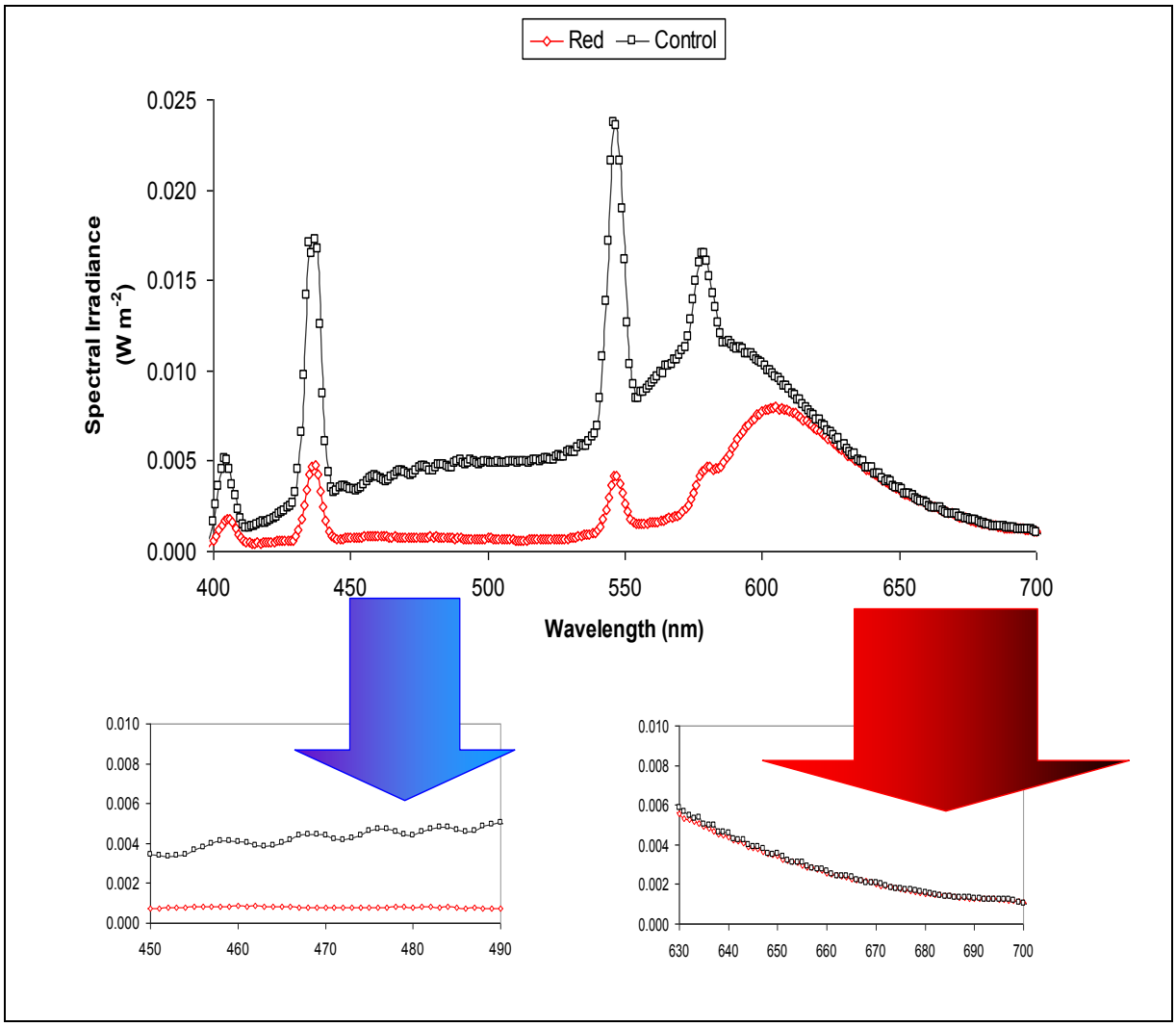

Figure 4.4 Spectral irradiance over the visible spectrum as recorded under control lighting conditions compared to spectral irradiance under the same light source, but beneath a red cellophane box. Those wavelengths that form the blue and red spectrums are shown enlarged.

4.3.3 Effect of red light on shoot elongation

The effect of red light on shoot elongation was not significant. However, the probability of the null hypothesis (no difference in means) for shoot elongation was low, whilst that for survival was substantially higher (Table 4.4). The difference between the two treatment means indicates a small, non-significant treatment trend. Red light shows a small, positive trend on shoot elongation (Figure 4.5).

Table 4.4 Summary of paired t-test performed on mean explant per plate elongation and on total survival per plate for the two light regimes tested.

	Elongation		Survival	
	Red	Control	Red	Control
Mean	0.933	0.600	1.700	1.200
Variance	0.316	0.440	1.122	1.067
Hypothesized Mean Difference	0		0	
d.f.	9		9	
s.e.	0.1792		0.3727	
t Stat	1.86		1.34	
P(T<=t)	0.096		0.213	

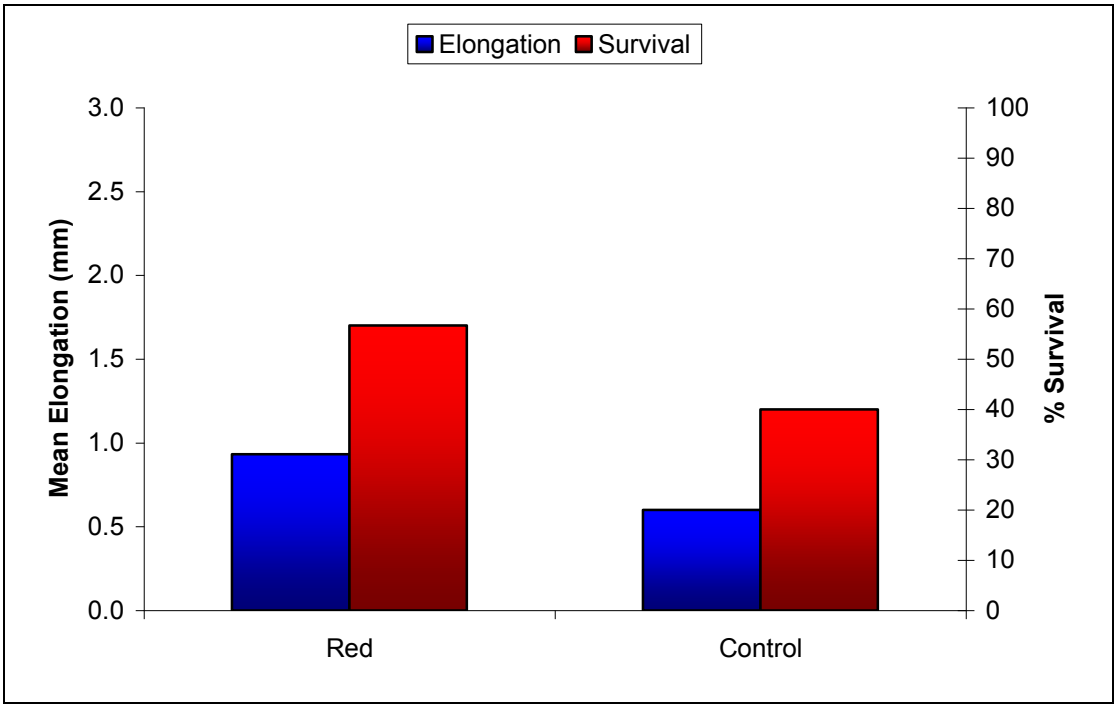

Figure 4.5 Mean explant elongation per plate and percentage survival per plate for the two light regimes tested.

4.4 DISCUSSION

The results show no significant effect of WPM on shoot elongation when compared to MS medium. This result indicates that there is no advantage to using WPM instead of

MS medium when attempting to elongate shoots produced through shoot apex culture of *Acacia mearnsii*. The results show no trends and the means are very similar, suggesting that this result is stable and not a result of experimental design.

Although no significant differences were observed in the results, some trends were noted and the effect of the red cellophane box on the culture light was encouraging. The red cellophane box reduced irradiance in all parts of the visible spectrum (400 – 700nm) except in the red spectrum (630 – 700nm) (Bohren and Clothiaux, 2006). This reduction had the effect of increasing the red proportion of the visible light spectrum and is best visualized when looking at the blue (450 – 490nm) and red wavelengths of the visible spectrum (Figure 4.3) (Bohren and Clothiaux, 2006). Red light has been shown to promote stem elongation in other species, whilst blue light has been reported as an elongation inhibitor (Appelgren, 1991; Nhut *et al.*, 2003). Therefore by successfully increasing the proportion of red light reaching the explants, the red cellophane box may be considered a viable, cheaper alternative to red light sources and filters.

The results show no significant effect of red light on shoot elongation of *Acacia mearnsii*. However, there was a weak trend indicating that red light does have a positive effect. This trend is backed up by other studies into the effect of red light on plant morphology (Appelgren, 1991; Moreira da Silva and Debergh, 1997; Nhut *et al.*, 2003). This trend was not significant, probably as a result of the relatively small sample size. This was due to the difficulties of setting up an elongation study with a suitably large number of regenerated shoots, whilst keeping genotypic effects to a minimum. If a larger and more robust experiment is designed, a more conclusive result should be attained.

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CHAPTER 5

EFFECTS OF PLANT HORMONES ON SHOOT ELONGATION OF *ACACIA MEARNSII* DURING SHOOT APEX CULTURE

ABSTRACT

For many species the important morphological and physical process of shoot elongation is often encouraged by the addition of plant hormones to the culture medium. The objective of this study was to test the effects of differing concentrations of various chemical additions to the culture medium on the shoot elongation of *A. mearnsii* during shoot apex culture. Two independent experiments were undertaken to compare differences in shoot elongation. Firstly using medium supplemented with various concentrations of GA_3 in comparison to the un-supplemented control, and secondly on medium supplemented with combinations of BA and IBA. Although results were not significant, shoots cultured with GA_3 showed decreased elongation and BA-supplementation showed some positive influences.

5.1 INTRODUCTION

The responses of an explant in culture are affected by the type of supporting medium, the temperature of incubation, the wavelength, intensity and duration of lighting conditions and the nutrient and growth hormone levels (Beck, 1999). These factors, both physical and chemical, combine in complex interactions and may have positive or negative effects on the growing explants. Therefore, the selection of chemical and hormonal factors plays a key role in the success or failure of any micropropagation technique. This selection does not only depend on the specific species traits and previous protocol selections, but also on the stage of development of the regenerating explant (Beck, 1999).

Shoot elongation is an important step in the successful micropropagation of any plant. It forms the important link between shoot initiation and root initiation (Beck *et al.*, 2000). Whilst shoot elongation has been achieved in *A. mearnsii* micropropagation, it is still problematic when using a small explant, such as those used in meristem or shoot apex culture, and is especially difficult when the explant is harvested from adult material (Huang *et al.*, 1994; Beck *et al.*, 2000). In the past, inducing shoot elongation during micropropagation of *A. mearnsii* has been attained through the addition of plant growth regulators and other supplements to the basal culture medium (Huang *et al.*, 1994; Correia and Graça, 1995; Quoirin *et al.*, 1998; Beck, 1999; Quoirin *et al.*, 2001). Benzyladenine (BA) and gibberellic acid (GA_3) have been demonstrated as suitable elongation hormones in the past, whilst indole butryic acid (IBA), which is more commonly used for root induction, has also shown some promise (Beck *et al.*, 2000; Quoirin *et al.*, 2001; Borges Júnior *et al.*, 2004). In this study the effects of differing concentrations of GA_3 and BA, combined with IBA, were individually evaluated for an effect on the shoot elongation of *A. mearnsii* during shoot apex culture.

5.2 MATERIALS AND METHODS

This study consisted of two separate elongation investigations. In order to carry out investigations on shoot elongation, it was first necessary to regenerate a large number of shoots. Since each plated explant does not necessarily develop into a regenerated shoot, a large number of shoot apexes needed to be cultured in order to produce enough regenerated shoots to study shoot elongation.

5.2.1 Plant material

Twelve three-year-old black wattle trees growing in 50 L black cultivation bags were utilised to provide explants. The maternal histories of these trees was known, but for simplicity are assigned individual codes C1 to C12. The trees were kept under shade cloth at the Institute for Commercial Forestry Research (ICFR) nursery and were watered regularly via an automated irrigation system to prevent the bags from drying out. Explants were harvested from the trees during 2005.

5.2.2 Selection and collection of shoot apexes

Healthy young shoots comprising of up to six shoot apexes were selected, resulting in a greater chance of mitotic division and thereby increasing the chance of regeneration during micro-propagation. These shoots were carefully removed, during the mid-morning, from a particular tree by hand and placed in a sterile petri dish, labelled with the corresponding tree identity. From each tree, 40 shoot apexes were collected and up to four individual trees were harvested on each collection day. Each tree was given a recovery time of at least two months between harvesting.

5.2.3 Topical sterilization

The harvested shoots were taken in sterile petri dishes directly from the nursery to the ICFR laboratory where they were exposed to a topical sterilisation process to remove any surface contaminants. All collected shoots underwent the following topical sterilisation process:

Step 1 Thoroughly wash under running tap water
Step 2 Surface sterilise in 70% ethanol for 1 min
Step 3 Soak in 0.2% Benlate® (benomyl 50% a.i.; Du Pont de Nemours, South Africa) for 10 min
Step 4 Rinse in distilled water
Step 5 Soak in 25% Jik (0.875% $NaOCl_2$) for 10 min
Step 6 Rinsed in distilled water
Step 7 Soaked in 500 mg L^{-1} ascorbic acid for 15 min

5.2.4 Dissection and shoot-initiation

Isolation of the shoot apexes through the dissection of the shoots was undertaken under a laminar flow bench with a sterile scalpel. The shoot apexes were then carefully placed on culture media using sterile tweezers.

Murashige and Skoog medium supplemented with 2.0 mg L^{-1} BA was chosen as the shoot-initiation medium as it was shown to be successful with *A. mearnsii* micropropagation in the past (Murashige and Skoog, 1962; Beck *et al.*, 1998; Beck, 1999; Beck *et al.*, 2000). The medium was supplemented with 30 g L^{-1} sucrose and

0.1 g L^{-1} Myo-inositol, altered to a pH of 5.8 and 3 g L^{-1} of Gelrite (solidifying agent) was added prior to the medium being autoclaved (at 121°C and 103kPa for 20 min). Each plate (65 mm sterile plastic petri-dishes from Labotec) contained four shoot apexes from the same mother plant and was sealed using parafilm. All cultures were maintained at a temperature of 25 ± 2°C, incubated under continuous light (23 µmol m^{-2} s^{-1}) with a photoperiod of 16 h light / 8 h dark. Cool white fluorescent tubes provided the light source. All culture protocols were accomplished using aseptic technique.

The apexes were monitored on a weekly basis until a suitable number of initiated shoots were available for further investigation. On each occasion that an explant became contaminated, the surviving explants were transferred to a new plate. Shooting was defined by a visual doubling in length of the explant, excluding callus growth. After sufficient numbers of shoots had been induced, callus tissue was carefully and aseptically excised and the remaining shoot was transferred onto shoot elongation medium.

5.2.5 Investigating GA_3

The addition of gibberellic acid (GA_3) to the elongation medium was investigated as a means of encouraging shoot elongation of *Acacia mearnsii* during shoot apex culture. Differing concentrations of GA_3 were added to MS medium and compared to the control (Table 5.1). All media were supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} Myo-inositol, altered to a pH of 5.8. Three g L^{-1} of Gelrite (solidifying agent) were added prior to the media being autoclaved (at 121°C and 103kPa for 20 min). The dissolved medium was then decanted into 125 ml glass bottles. Ten sterile 125 ml glass bottles each containing three regenerated shoots (30 in all) were used for each treatment. The mother plant identity for each shoot was known and recorded by numbering each shoot position on the outside of each glass bottle. Treatments had identical mother plant proportions (Table 5.1).

Table 5.1 Summary of explant source, showing individual mother plant compositions for all treatments investigated and the plant hormone additives for each treatment.

Bottle No.	Treatment 1a	Treatment 2a	Treatment 3a	Treatment 4a	Treatment 5a
	Control (MS)	MS + 0.5 mgL^{-1} GA_3	MS + 1.0 mgL^{-1} GA_3	MS + 1.5 mgL^{-1} GA_3	MS + 2.0 mgL^{-1} GA_3
1	3 C2	3 C2	3 C2	3 C2	3 C2
2	2 C2; 1 C5	2 C2; 1 C5	2 C2; 1 C5	2 C2; 1 C5	2 C2; 1 C5
3	3 C4	3 C4	3 C4	3 C4	3 C4
4	2 C4; 1 C5	2 C4; 1 C5	2 C4; 1 C5	2 C4; 1 C5	2 C4; 1 C5
5	2 C6; 1 C3	2 C6; 1 C3	2 C6; 1 C3	2 C6; 1 C3	2 C6; 1 C3
6	2 C7; 1 C3	2 C7; 1 C3	2 C7; 1 C3	2 C7; 1 C3	2 C7; 1 C3
7	C7; C8; C9	C7; C8; C9	C7; C8; C9	C7; C8; C9	C7; C8; C9
8	3 C11	3 C11	3 C11	3 C11	3 C11
9	3 C12	3 C12	3 C12	3 C12	3 C12
10	3 C12	3 C12	3 C12	3 C12	3 C12

All cultures were maintained at a temperature of 25 ± 2°C, incubated under continuous light (23 µmol m^{-2} s^{-1}) with a photoperiod of 16 hr light / 8 hr dark. Cool white fluorescent tubes provided the light source and cultures were monitored on a weekly basis for 14 d. All culture protocols were performed using aseptic technique.

5.2.6 Investigating BA and IBA

Indole-3-butyric acid (IBA) and 6-benzyladenine (BA) were investigated as a means of encouraging shoot elongation of *Acacia mearnsii* during shoot apex culture. Differing concentrations of BA and IBA were added to MS medium and compared to the control (Table 5.2). All media were supplemented with 30 g L^{-1} sucrose and 0.1 g L^{-1} Myo-inositol, altered to a pH of 5.8. Three g L^{-1} of Gelrite (solidifying agent) was added prior to the media being autoclaved (at 121°C and 103kPa for 20 min). Ten sterile 125 ml glass bottles each containing 3 regenerated shoots (30 in all) were used for each treatment. The mother plant identity for each shoot was known and recorded by numbering each shoot position on the outside of each glass bottle. Treatments had identical mother plant proportions (Table 5.1).

Table 5.2 Summary of explant source, showing individual mother plant compositions for all treatments investigated and plant hormone additives for each treatment.

Bottle No.	Treatment 1b	Treatment 2b	Treatment 3b	Treatment 4b	Treatment 5b
	Control (MS)	MS + 2 mgL^{-1} BA	MS + 2 mgL^{-1} BA + 0.01 mgL^{-1} IBA	MS + 2 mgL^{-1} BA + 0.1 mgL^{-1} IBA	MS + 2 mgL^{-1} BA + 1.0 mgL^{-1} IBA
1	3 C9	3 C9	3 C9	3 C9	3 C9
2	3 C9	3 C9	3 C9	3 C9	3 C9
3	3 C9	3 C9	3 C9	3 C9	3 C9
4	3 C9	3 C9	3 C9	3 C9	3 C9
5	2 C11; 1 C9	2 C11; 1 C9	2 C11; 1 C9	2 C11; 1 C9	2 C11; 1 C9
6	3 C1	3 C1	3 C1	3 C1	3 C1
7	3 C1	3 C1	3 C1	3 C1	3 C1
8	3 C10	3 C10	3 C10	3 C10	3 C10
9	3 C10	3 C10	3 C10	3 C10	3 C10
10	3 C10	3 C10	3 C10	3 C10	3 C10

All cultures were maintained at a temperature of 25 ± 2°C, incubated under continuous light (23 µmol m^{-2} s^{-1}) with a photoperiod of 16 hr light / 8 hr dark. Cool white fluorescent tubes provided the light source and cultures were monitored on a weekly basis for 14 d. All recipes of solutions have been taken up in the appendix and all culture protocols were accomplished using aseptic technique

5.2.7 Monitoring and quantification of results

In order to measure shoot elongation during the two week elongation phase, a sterile system of measuring shoot length was devised. A ruler showing millimetre detail was photocopied onto plastic and this plastic was then thoroughly washed in 70% ethanol and placed on the laminar flow bench. A sterile glass petri-dish was then placed on top of the ruler and measurements were taken on this. Each shoot was measured before the elongation phase and this measurement together with its position in the jar and its parent tree was noted. After two weeks the length of each shoot was again measured and this, together with the condition of the explant, was noted.

5.2.8 Statistical analysis

Statistical analysis was carried out using Genstat Version 9.1. The mean shoot elongation of explants per plate was calculated and treated as one data point, as was each plate's survival count. Data was transformed according to the following linear transformation: $y = x + 10$; where x is the original data and y the transformed data. A one-way (shoot elongation) ANOVA without blocking, but with the addition of two covariates (plate composition (mother plants); plate survival count), was performed on the transformed data and the results were recorded. This analysis was performed on both data sets.

5.3 RESULTS

Results are shown without transformations, unless specified, because the linear transformation did not alter significance levels, only the co-efficient of variation. The results of both the one-way ANOVA analyses (GA_3, and BA + IBA combinations) showed no significant treatment effects (Table 5.3). The effect of the two covariates (plate composition (mother plants) and plate survival count) showed differing levels of significance across both investigations (Table 5.3).

Table 5.3 Summary of the analyses of variance performed on mean explant elongation per plate and on total survival per plate for both GA_3 and BA and IBA combinations. All data was transformed according a linear transformation prior to analysis.

		GA_3			BA and IBA		
Source of variation							
	d.f.	F pr.	m.s.	l.s.d.	F pr.	m.s.	l.s.d.
Treatment	4	0.604	1.858^{NS}	1.505	0.125	0.7648^{NS}	0.5703
Covariates:	2	0.041	9.317^{*}		< .001	7.8708^{***}	
- Plate	1	0.110	7.201^{NS}		0.003	4.1084^{**}	
- Survival	1	0.046	11.434^{*}		< .001	11.6332^{***}	
Residual	43		2.7			0.3985	
Total	39						
Summary of data							
s.e.		1.643			0.6313		
cv%		14.3			5.6		

*** Significant at $P<0.001$

** Significant at $P<0.01$

* Significant at $P<0.05$

^{NS} Not significant

5.3.1 Effect of GA_3 on shoot elongation

The effect of GA_3 on shoot elongation was not significant. The difference between the treatment means was small with no treatment trends observed (Figure 5.1). However, the covariate effect of explant survival on shoot elongation was significant (Table 5.3).

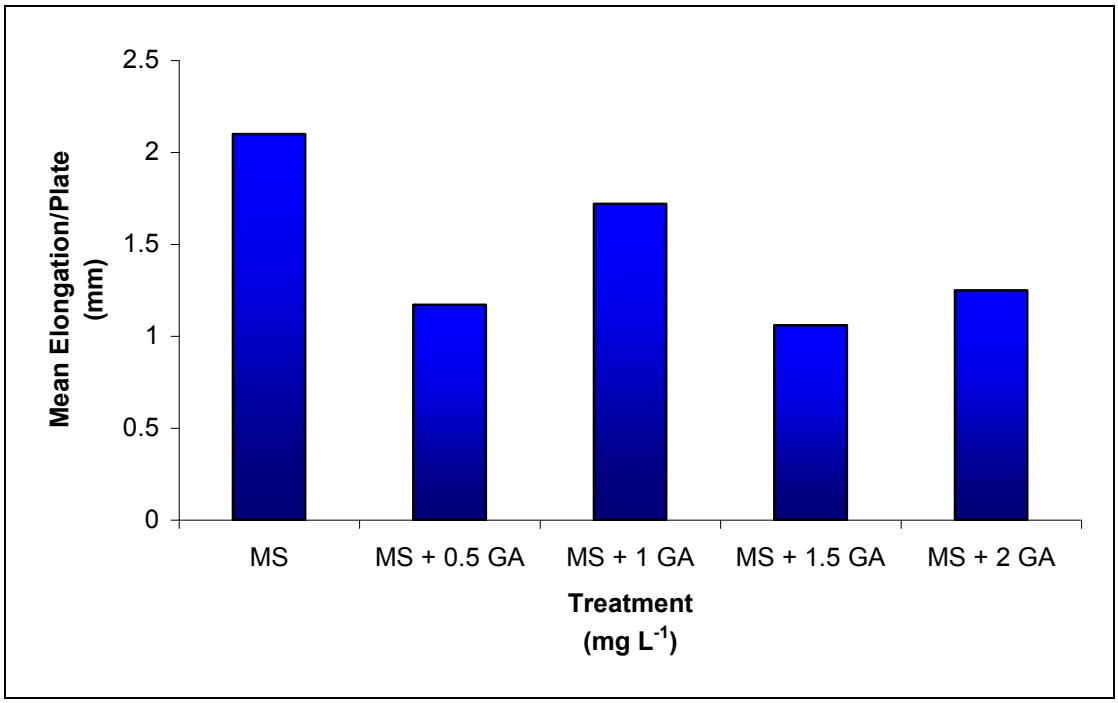

Figure 5.1 Mean explant elongation per plate for the all treatments tested.

5.3.1 Effect of BA and IBA combinations on shoot elongation

The effect of BA and IBA combinations on shoot elongation was not significant. However, the differences between the treatment means indicated a small, non-significant treatment trend. Some BA and IBA combinations showed a small, positive trend on shoot elongation (Figure 5.2). Again, the covariate effects of explant survival and plate composition on shoot elongation was significant (Table 5.3).

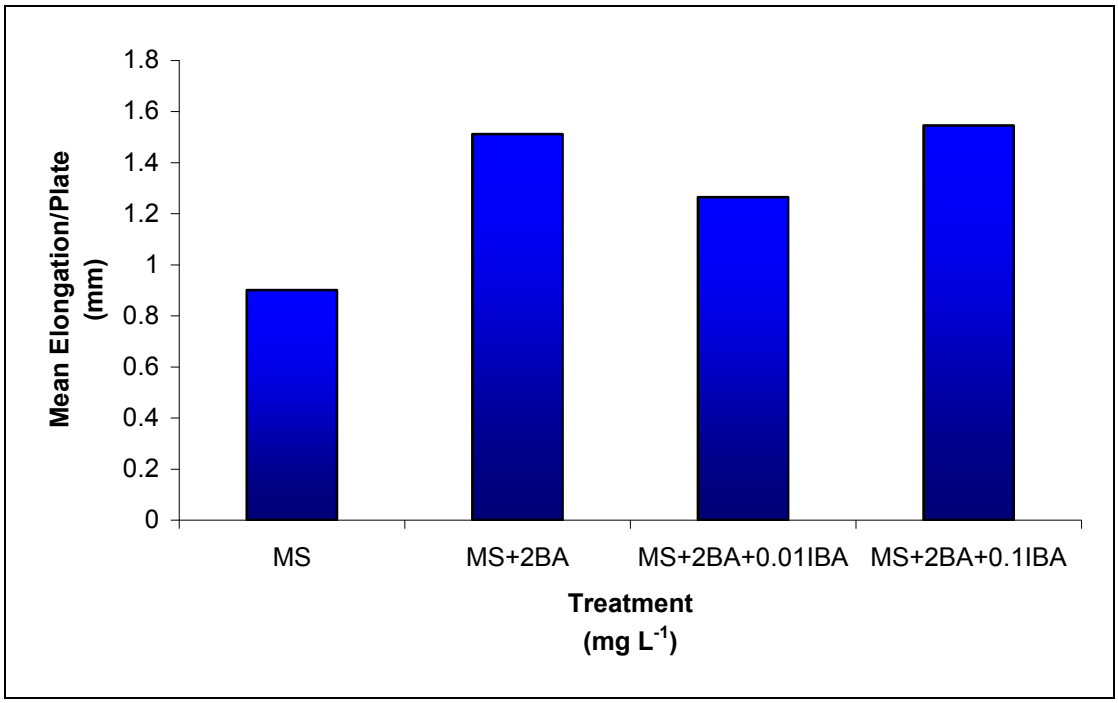

Figure 5.2 Mean explant elongation per plate for the all treatments tested.

5.4 DISCUSSION

Although there were no significant treatment differences observed in the results, some trends were noted and most of the covariate effects were significant (Table 5.3). The covariate effect of survival on shoot elongation for both investigations was significant (Table 5.3). This result was not unexpected because an unhealthy or dying shoot will not be able to elongate and therefore negatively influences the mean elongation of each plate. The covariate effect of plate composition on shoot elongation for the BA and IBA combinations investigation was significant (Table 5.3). This result supports the results of Chapter Three where mother plant was shown to have a significant influence on the response of *Acacia mearnsii* explants to shoot apex culture.

Although there were no significant differences between the control and GA_3 treatments, and no clear trends could be ascertained, the reduction in shoot elongation of all four GA_3 treatments relative to the control indicated that GA_3 may negatively affect shoot elongation (Figure 5.1). This is in contrast to previous reports where it was suggested that GA_3 may have a positive effect on the elongation of

Acacia mearnsii shoots during micropropagation (Beck *et al.*, 2000). A small, non-significant positive trend for some BA and IBA combinations on shoot elongation was indicated by some relatively large differences in the treatment means (Figure 5.2).

The results indicated that some BA and IBA combinations have a positive effect on shoot elongation and if a larger and more robust experiment were designed, then a more conclusive result might be attained. Some previous studies suggested that the BA concentration should be lowered to encourage elongation (Correia and Graça, 1995). As the positive effects of BA on *Acacia mearnsii* micropropagation are well documented, no more resources should be allocated to this line of study (Beck *et al.*, 2000; Quoirin *et al.*, 2001; Borges Júnior *et al.*, 2004). In contrast the poor performance of GA_3 is surprising given other findings which suggested GA_3 is a suitable plant hormone for the promotion of shoot elongation (Beck *et al.*, 2000). However, both these findings must be viewed in context of their lack of statistical significance and are not deemed conclusive.

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CHAPTER 6

THESIS OVERVIEW

6.1 INTRODUCTION

Research into the micropropagation of black wattle in South Africa is regarded as important for two reasons. Firstly, micropropagation technology allows plant breeders to select and propagate mature tissue, which in turn allows for complete capture of selected traits (Jones and van Staden, 1997). Secondly it may solve the problem posed by the highly invasive nature of black wattle. If research into the sterilisation of black wattle is successful, then foresters will have to rely on clonal propagation to supply material for their growing operations (Beck and Dunlop, 2001). Therefore as part of the Institute for Commercial Forestry's *Acacia mearnsii* vegetative propagation programme, the main focus of this research was to overcome various problems associated with direct organogenesis of *ex vitro* material. Ultimately, it is hoped that a viable protocol for shoot apex culture of mature black wattle can be achieved. In order for this goal to be attained, various problems must be eliminated. One such problem is the resistance shoots show to elongation once induced (Beck *et al.*, 2000). Surface sterilisation of *ex vitro* grown black wattle explants, whilst not identified as a problem, has in the past made use of mercuric chloride, an environmentally dangerous chemical (Saha, 1972). The aims of this study were therefore:

1. To investigate the effects of different sterilisation agents and exposure times on cultured *Acacia mearnsii* shoot apex contamination levels.

2. To test the effects of harvest season and individual mother plant on shoot initiation during shoot apex culture of *Acacia mearnsii*.

3. To investigate the effects of physical and hormonal factors on shoot elongation of *Acacia mearnsii* during shoot apex culture.

6.2 SCREENING OF STERILISATION AGENTS AND EXPOSURE TIMES

6.2.1 Experimental approach and outcomes

The objective of this research was to investigate alternative sterilisation agents to the dangerous chemical, mercuric chloride ($HgCl_2$), and to determine the effect of sterilant exposure time on shoot apex culture of *Acacia mearnsii*. Healthy young shoots from several different trees were harvested and exposed to differing topical sterilisation processes. Shoot apexes were then excised and cultured on MS medium supplemented with 2.0 mg L^{-1} BA. Explants were monitored for 21 d for signs of contamination and shooting. Household bleach (NaOCl), diluted 1:3 in water, resulted in decontaminated explant levels not significantly different to the 0.2% $HgCl_2$ treatment and resulted in shooting levels significantly higher than the 0.2% $HgCl_2$ treatment. Furthermore household bleach is handled in an easier manner than $HgCl_2$ and, at low concentrations, it seems to cause smaller phytotoxic effects. There was no significant effect of sterilant exposure time on decontaminated explant levels, whilst the shortest exposure time resulted in significantly higher shooting levels than the other two times tested.

6.2.2 Future research

Whilst the importance of household bleach (NaOCl), diluted 1:3 in water, as a viable surface sterilant alternative to 0.2% $HgCl_2$, should not be overlooked, it does not address the problem of endogenous contamination often reported in micropropagation techniques (Ferrador *et al.*, 2005; Rajesh and Anil, 2006). Although the use of the shoot apex region as an explant limits the impact of these microorganisms, the problem persists. Further research on sterilisation should focus on these endogenous contaminants. Two possible research avenues are recommended. Firstly the endophytic microorganisms should be isolated out of contaminated cultures and identified, allowing

for a suitable antimicrobial or fungicidal agent to be added to the culture medium in small concentrations. The second avenue involves the identification of a more systemic sterilant, which penetrates within the explant. This approach has yielded rewards in other species (Rajesh and Anil, 2006). The successful control of endogenous contaminants would allow the use of more robust explants which have been shown to be better suited to the stresses of micropropagation (Bonga and von Aderkas, 1992).

6.3 SEASONAL AND MOTHER PLANT EFFECTS

6.3.1 Experimental approach and outcomes

The objective of this research was to determine the effect of two *ex vitro* variables, season and mother plant, on shoot apex culture of *Acacia mearnsii*. Explants from four individual mother plants were cultured on MS media supplemented with 2.0 mg L^{-1} BA during four separate seasons and monitored for 21 d for signs of contamination and shooting. Spring was found to be the best harvesting season as spring explants showed significantly higher decontaminated explant levels and shooting levels than explants harvested in the three other seasons. The effect of mother plant (genotype) on the performance of *Acacia mearnsii* explants during shoot apex culture was also found to be significant, especially with regard to shooting levels.

6.3.2 Future research

The significant effects of season and mother plant on shoot apex culture of *Acacia mearnsii* provide some useful answers and pose some difficult questions leading to possible research avenues. Firstly it is obvious that spring is the best time for explant harvesting. This may be due to environmental factors influencing the growth of infecting microbes or the health of the plant itself, or, more likely, a combination of both (Siril and Dhar, 1997; Bhatt and Dhar, 2004). Research should be conducted into the physiological state of the mother tree during this optimum harvesting time. In other species there is an internal control within the plant that mobilises resources toward the

shoot apical meristem in early spring and it is probable that a similar mobilisation of resources in *Acacia mearnsii* positively influences its shoot apex culture during spring (Damascos *et al.*, 2005). The identification of these particular resources would allow researchers to manipulate the mother plant conditions or to alter the explant culture additives to better simulate spring conditions and thus extend the optimal time frame for explant harvesting. Secondly, the results indicate that shooting may be influenced by the genotype of the mother plant. This hypothesis, although reported in other species, requires further validation for *Acacia mearnsii* (Banerjee *et al.*, 2007). The significant differences observed between mother plants may be due to the differing environmental conditions from bag to bag or may be due to genotypic responses to tissue culture. Further research should be focussed on family effects due to the scarcity of clonal *Acacia mearnsii* material. The results of both these avenues of research would aid in the understanding of mother plant environment and genotype effects during the micropropagation of *Acacia mearnsii* and lead to a better manipulation of these factors.

6.4 HORMONAL AND PHYSICAL EFFECTS ON SHOOT ELONGATION

6.4.1 Experimental approach and outcomes

The objective of this study was to test the effects of an alternative basal medium, red light and differing concentrations of chemical additions to the culture medium on shoot elongation of *Acacia mearnsii* during shoot apex culture. Four independent experiments were undertaken comparing: shoot elongation on Woody Plant Medium (WPM) (Lloyd and McCowan, 1981) to the Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium control; shoot elongation under a red cellophane box compared to control culture light conditions; shoot elongation on media supplemented with various concentrations of GA_3 to the un-supplemented control and shoot elongation on media supplemented with combinations of BA and IBA compared to a control. In order to carry out investigations on shoot elongation it was first necessary to regenerate a large number of shoots. Since each plated explant does not necessarily develop into a regenerated shoot, a vast amount of shoot apexes needed to be cultured in order to

produce enough regenerated shoots to study shoot elongation. In order to measure shoot elongation during the two week elongation phase a sterile system of measuring shoot length had to be devised. Shoot lengths were measured aseptically before and after an elongation period to determine the true elongation of each initiated shoot.

Although no significant effects were observed, many trends were noted and red cellophane successfully increased the proportion of red light reaching the explants. The results indicate that there is no advantage to using WPM instead of MS medium when attempting to elongate shoots rejuvenated through shoot apex culture of *Acacia mearnsii*, whilst the effect of GA_3 showed a negative trend. The effects of red light and some BA and IBA combinations showed positive trends on the elongation of initiated shoots.

6.4.2 Future research

The treatment effects were not significant for any of the four experiments, probably as a result of a non-robust experimental design. The sample size was small, due to the difficulties of setting up an elongation study with a suitably large number of regenerated shoots, whilst keeping the genotype effect to a minimum. This opens clear avenues to extend this research. However the effects of basal media and GA_3 showed no positive trends on shoot elongation and therefore resources should rather be allocated to other investigations. This would enable a more focused study on the effects of red light and BA on shoot elongation of *Acacia mearnsii*. The interaction effects of red light together with BA could also be researched in one stand-alone experiment, thereby limiting the number of regenerated shoots required. Another research possibility is the investigation of the effect of red cellophane compared to more expensive red light sources and filters shown to promote stem elongation in other species, (Appelgren, 1991; Nhut *et al.*, 2003). The continued research into these two promising promoters of shoot elongation could help form the link between shoot initiation and root initiation and lead to a successful micropropagation protocol for mature A*cacia mearnsii* material (Beck *et al.*, 2000).

6.5 OVERALL CONCLUSION

Acacia mearnsii remains an important species in the economics of South Africa's forestry industry. However if *Acacia mearnsii* yields are to remain competitive with other forestry species, the large genetic gains of the past must be maintained. *Eucalyptus* species have illustrated the advantages of large-scale clonal planting and in order for *Acacia mearnsii* to experience similar advantages, a viable protocol for the cloning of this species must be attained. Economics is not the only reason for this protocol, from an environmental perspective, the future deployment of sterile black wattle relies on clonal propagation.

This investigation successfully addressed some of the problems associated with micropropagation of *Acacia mearnsii*. Shoot apex culture shows promise and further research into this technique should be considered. A viable surface sterilant alternative to mercuric chloride was successfully identified. This alternative is not only safer to use but shows a large reduction in its phytotoxic effects. The effects of season and mother plant on shoot apex culture was successfully investigated, resulting in a better understanding of mother plant influences on tissue culture as well as the identification of an optimum time frame for explant selection. Finally two possible shoot elongation promoters were identified for further research and a cheap alternative to red light sources and screens was identified.

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APPENDIX 1

BASAL MEDIA INGREDIENTS

Macronutrients			
Name	**Formula**	**Murashige and Skoog Medium Concentrations (mg L^{-1})**	**Woody Plant Medium Concentrations (mg L^{-1})**
Ammonium nitrate	(NH_4NO_3)	1650	400
Boric Acid	(H_3BO_3)	6.2	6.2
Calcium chloride	($CaCl_2 \cdot 2H_2O$)	440	96
Calcium nitrate	(Ca(NO3)2*4H2O	0	556
Cobalt chloride	($CoCl_2 \cdot 6H_2O$)	0.025	0
Cupric sulfate	($CuSO_4 \cdot 5H_2O$)	0.025	0.25
Ferrous sulfate	($FeSO_4 \cdot 7H_2O$)	27.8	27.8
Magnesium sulfate	($MgSO_4 \cdot 7H_2O$)	370	370
Manganese sulfate	($MnSO_4 \cdot 4H_2O$)	22.3	22.3
Potassium iodine	(KI)	0.83	0
Potassium nitrate	(KNO_3)	1,900l	0
Potassium phospate	(KH2PO4)	0	170
Potassium sulfate	(KH_2PO_4)	170	990
Sodium molybdate	($Na_2MoO_4 \cdot 2H_2O$)	0.25	0.25
Zinc sulfate	($ZnSO_4 \cdot 7H_2O$)	8.6	8.6
	$Na_2EDTA \cdot 2H_2O$	37.2	37.2
Common additives			
Myo-inositol		100	100
Nicotinic Acid		0.5	0.5
Pyridoxine*HCl		0.5	0.5
Thiamine *HCl		1.0	1.0
Glycine		2.0	2.0
Agar		10 000	6 000
Sucrose		30 000	30 000

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