IN-VITRO PROPAGATION OF MMUPUDU (*Mimusops zeyheri*) FRUIT TREE

YVONNE MMATSHELO MAILA

A

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

MASTERS IN AGRICULTURAL SCIENCE

SCHOOL OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES
DEPARTMENT OF PLANT PRODUCTION
UNIVERSITY OF THE NORTH
PRIVATE BAG X1066
SOVENGA
0727

SUPERVISOR: Dr. P. W. MASHELA
CO-SUPERVISOR: Dr. B. N. OKOLE

2001

[redacted]

[redacted]

A

special dedication to

JOHANNES MAKAMASI MAILA

my father

for his passion in growing plants, his love and support made me to reach this far in my studies.

(1945 – 1997)

TABLE OF CONTENTS

DECLARATION

As far as I am aware, this work is original by the candidate and all non-original work have been properly acknowledged.

CANDIDATE: MAILA MMATSHELOYVONNE

SIGNATURE: _____ DATE: 03.06.02

SUPERVISOR: DR P.W. MASHELA

SIGNATURE: _____ DATE: 26-09-2002

CO-SUPERVISOR: DR B. N. OKOLE

SIGNATURE: _____ DATE: _____

ACKNOWLEDGEMENTS

This study could never have been completed without the help of Dr. B. N. Okole, who accepted responsibilities so abruptly; when he was called upon to assist with the facilities at African Biotechnologies. The supervision, motivation, guidance and patience he willingly contributed towards the training of the author were indispensable. Also, the allowance offered made a great difference.

Dr. P. W. Mashela encouraged and supported the study in various ways, without which the study could not have been successfully completed. The horticultural and research training and the scholarship are greatly appreciated.

The author thanks her mother, Lillian Maila and her family, who were always there for her. To God be all the glory.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Micropropagation of Mmupudu (*Mimusops zeyheri*) using the shoot tip culture technique was successfully developed. Cultures were initiated from the meristem shoot tips obtained from Mmupudu plantlets raised in the greenhouse. Shoot tip explants cultured on Murashige and Skoog basal salt medium (MS) with macro and micro elements at half strength supplemented with 0.57 μM 3-indolyl-acetic acid (IAA) and 8.87 μM 6-benzyladenine (BA) resulted in establishment of 80% explants. Out of the three cytokinins tested, 6-benzyladenine (BA) and 6- furfuryl aminopurine (kinetin) were the most effective in shoot proliferation. An average of 2.05 shoots per explant were obtained when BA was used at 8.87 μM in combination with kinetin at 9.29 μM. Half strength MS medium supplemented with 9.80 μM indol-3-butyric acid (IBA) and activated charcoal stimulated 90% excised shoots to initiate adventitious roots. Rooted shoots with fully expanded leaves were weaned to the greenhouse and nursery, with survival rates of 60% and 50%, respectively. Reasons for low hardening rates are not clear, thus future research should focus on hardening from culture vessels to greenhouse and nursery conditions.

Chapter 1

INTRODUCTION

Mmupudu (*Mimusops zeyheri;* English = Transvaal Red Milkwood; Afrikaans = Moepel) is an indigenous woody perennial tree that grows in semi-arid regions of the Northern Province. The natural habitat of the tree is mainly on mountain hillsides, where the soil is shallow. This tree developes non-aggressive root systems, with various attributes that are associated with drought tolerance (Venter and Venter, 1996). The tree is evergreen, with a round shaped crown, and grows to a height of 15 m.

Smallscale farmers were historically settled in areas where soil is marginal for the cultivation of most exotic fruit crops that require soils that are at least 1 m deep. Mmupudu trees are of economic importance and both fruit and seedlings are in demand (Mashela and Mollel, 2001).

The wood is good for general-purpose timber, with selected planks making strong benches and chairs (Venter and Venter, 1996). Vitamin C content, ranging from 50 to 80 mg/g fresh fruit, is the highest of any edible fruit. Currently, citrus remains the major source of vitamin C in the Northern Province, but a large content of vitamin C is contained in the inedible peel. Also, citrus is sensitive to drought stress and it has therefore, not been popular as a garden tree in rural areas. Another attribute for Mmupudu fruits is that they ripen in late spring, when there are hardly any other mature fruits on trees.

Due to the climatic conditions that prevail in the Northern Province, water shortage is known to be a major problem affecting fruit production. Most farmers in the Northern Province depend on borehole water for irrigation. Salty borehole water used for irrigation reduces plant growth and fruit yield and increases densities of certain pests like the citrus nematode, T*ylenchulus semipenetrans*, (Mashela, 1992). Farmers in the Northern Province rated Mmupudu as being the most suitable species for domestication and commercialization (Mashela and Mollel, 2001). Also, some farmers indicated that they were willing to farm with this fruit tree on a small scale; whereas others indicated that they needed it as a garden tree. Because Mmupudu is drought tolerant, much less water would be required for irrigation when compared with exotic fruit trees under the semi-arid conditions of the Northern Province.

Domestication of Mmupudu is important as it has characteristics which render this tree the most suitable as an alternative crop for smallholder farming systems in the Northern Province. Its domestication may enhance food security and the establishment of a sustainable agro-ecosystem to combat soil erosion. However, the major constraint in growing this crop by smallscale farmers is the unavailability of affordable seedlings. Sexually produced seedling plants of Mmupudu are genetically variable and grow slowly. Consequently, Mmupudu seedlings are expensive (Mashela and Mollel, 2001). The objective of this study

is to develop a tissue culture technique that will be used to mass-produce affordable and healthy Mmupudu plantlets within a reasonable period of time.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

Conventional propagation of Mmupudu (*Mimusops zeyheri*) fruit trees can be achieved from seeds. Germination percentage of fungicide-treated Mmupudu seeds is 60-80% (Venter and Venter, 1996). The first true leaf emerges after 6 weeks and it is at this stage that the plants are suitable for transplanting into black nursery plastic bags. Although the tree is drought tolerant and can take certain amount of cold, it is also frost-tender, it grows either in the sun or shade (Palgrave, 1977).

Mmupudu is a slow grower (Venter and Venter, 1996). The slow growth of this plant may delay attainment of the reproductive stage. Sexually propagated fruit trees take a long time before reaching maturity (Hartmann *et al.*, 1981). The juvenile and transitional stages of sexually propagated Mmupudu fruit trees are long (10-20 years). Plants do not flower during the juvenile stage, whereas during the transitional stage flowering is erratic. Thus, most sexually propagated fruit trees reproduce in 10 to 20 years after planting. Also, sexually propagated plants are known for their high degree of genetic and phenotypic variations (Lisowska and Wysokinska, 2000).

2.2 Need for an unconventional method of propagation

The long period needed to attain reproductive maturity in fruit trees is a serious obstacle in their commercialization. Because high capital is required in initiating an orchard, long unproductive periods are uneconomic. A system that would allow for clonal propagation of Mmupudu, would eliminate the juvenile and transitional stages. The application of micropropagation techniques for propagation of Mmupudu would provide clonal rootstocks of homogenous characteristics (Vieitez *et al.*, 1993; Meier and Reuther, 1994; Chalupa, 1996). Because micropropagated plants have short juvenile and transitional stages when obtained from mature parts of the plants, full reproduction would be attained in less than five years (Hartmann *et al.*, 1981).

2.3 Micropropagation approach

Micropropagation is the art or science of growing plant cells, tissues or organs isolated from the mother plant on artificial nutrient media under aseptic conditions. Micropropagation is feasible due to the ability of a single cell to develop into a plant, which is referred to as totipotency. Prior to laboratory execution of the techniques, few prerequisites are necessary.

2.3.1 Selection of sources of explants

Selecting a suitable source of explant material is essential for the success of micropropagation. Results obtained in micropropagation can be influenced by the way the mother plant is treated and by the environment in which it is growing. George (1993) reported that cultures could be initiated with clean and disease-free material from genetically uniform plants. Mother plants growing in the field are subject to higher rates of contamination and a more variable environment than those growing under greenhouse conditions (Dixon, 1986).

2.3.2 The age of explants

Plant age and the degree of differentiation of tissues are often interrelated and produce inter-active effects in tissue culture. In most woody plants, adventitious roots are produced more readily on juvenile explants than on those derived from more mature trees (Hartmann *et al.*, 1981). The use of such explants has been effective in permitting regeneration in woody plants where there has been no success with other tissues.

2.3.3 The size of explants

Optimum sizes for explants used to initiate tissue cultures range from 10 to 30 mm long with apices or stem sections carrying lateral buds. Such explants can sometimes be more readily disinfected and are frequently more easily established *in-vitro* (George, 1993). Davies and Dale (1979) reported that smaller explants, whether originating as shoot tips or fragments of whole plant tissues, hardly survive in cultures. However, larger explants may be difficult to decontaminate effectively. The two-noded segments of Barberry (*Berberis thumbergii)* produced more shoots per bud than single node explants (Uno and Preece, 1987). Growth of Pistachio nut tree (*Pistacia spp.*) explants bearing three axillary buds was greater than those with two or one bud (Barghchi, 1986).

2.4 Problems in initiating and maintaining of cultures

Proper initiation and maintaining of cultures is essential for the success of micropropagation. Exudation of phenols by the explants into the media, results in the decline of vigour in culture. Also, should necessary precautions not be taken, shoot tip necrosis might affect explant growth and development.

2.4.1 Blackening or browning

Cut surfaces of most explants start to discolour soon after excision due to exudation of phenols. Explants, frequently continue to darken when they are introduced into the culture vessels, where they may also exude dark coloured substances into the medium. Not all substances produced are inhibitory, but it is frequently found that once discolouration occurs growth is inhibited and tissues may die unless remedial steps are taken (George, 1993).

Damage from the production of dark pigments is usually most severe during the initial stages of a culture and ceases to be a problem once explants have commenced growth (Garton and Moses, 1986). Young juvenile tissues are often less prone to browning on excision than older ones, as demonstrated with Rosa 'Paul's Scarlet' (Muhitch and Fletcher, 1984). Succulent tissues excised from heavily pruned and etiolated shoots of woody plants are often more liable to browning than those taken from adult tissues because the former exude more phenols into the medium.

Minimizing the extent to which explants of susceptible plants are wounded or damaged during excision and sterilization can reduce browning. In some species, the sterilants used to decontaminate explants can be responsible for accentuating the browning of explants. Where browning presents a severe problem, it is advisable to replace one sterilant solution with the other. Browning

of Bird of paradise (*Strelitzia reginae*) was less severe when 0.3% mercuric chloride was used as a sterilant instead of 9% calcium hypochlorite (Ziv and Halevy, 1983). Other commonly used methods of reducing browning include regular transfer of explants into fresh medium, use of activated charcoal and addition of anti-oxidants like citric acid, ascorbic acid and dithiotheltol in the growing medium (Dr. Blessed Okole-Pers. Com., 2001).

2.4.2 Decline in vigour of shoot cultures

George (1988) observed a decline in vigour during the initiation stage of Walnut (*Juglans regia*). Shoots showed chlorosis, leaf abscission and retarded growth during their first few months of culture. Frequent transfer of the cultures into fresh medium and trimming explant bases alleviated the problem. Sub-culturing ahead of time can sometimes prevent degeneration. In Red-flowering gum tree (*Eucalyptus ficifolia*) cultures, plants had normal growth for two months and then later on, declined rapidly (De Fossard and Bourne, 1976). Sub-culturing to a fresh medium resolved the problem.

2.4.3 Shoot tip necrosis in cultures

Necrosis describes the death of living plant tissues in an organ (Hartmann *et al.*, 1981). Its occurrence on a portion of an explant commonly leads to the death of the whole explant.

Apical necrosis occurs in shoot cultures, especially in woody plants. Although there may be other causes, the most common cause for apical necrosis is Ca deficiency in the apices of cultured shoots due to the ion either having been inadequately absorbed from the medium, or not having been transported (Debergh, 1988; George *et al.*, 1988). Calcium deficiency in plants results in poor root growth and in the blackening and curling of the margins of apical leaves. Cessation of growth and death of shoot tips often follows. Experiments have been carried out to show that a shortage of this element is associated with shoot tip necrosis in *Betula, Populus* and other woody plants (Debergh, 1988). An inadequate supply of Ca can result from limited Ca uptake, and inadequate transport, the latter being caused by the absence of a transpiration stream due to high humidity in culture vessels.

Shoot tip necrosis can be alleviated by increasing the concentration of Ca in the medium or by changing the physical environment in which the cultures are grown so that transpiration is increased (McCown and Sellmar, 1987). The occurrence of necrosis can also be reduced by frequent sub-culturing into fresh medium or by adding calcium nitrate, calcium gluconate or calcium chloride to the medium (Samir Debnath-Pers com., 2001).

2.5 Contamination and de-contamination procedures

Plants are almost always infected externally with fungi, bacteria and yeast (George, 1993). These organisms may be present on all external surfaces and are also likely to find their way into small crevices, such as those between the bud scales and under ligules or stipules. In tissue culture, it is necessary to remove external microorganisms from explants with chemicals and disinfectants before culturing *in-vitro* (Dixon, 1986). Bacteria and fungi are able to grow readily on plant culture media that contain sugars, amino acids and vitamins.

2.5.1 Surface sterilization

Surface sterilization can be carried out with various germicidal reagents. The most commonly used materials are those containing sodium or calcium hypochlorite and simple alcohol, particularly ethanol (Dychdala, 1977). Sodium hypochlorite (NaOCl) is soluble in water. Although aqueous solutions can be obtained from commercial suppliers, most laboratories use household or industrial bleaches (e.g. *Domestos*®) as a convenient source since it is cheaper and readily available.

Calcium hypochlorite ($CaO(Cl)_2$) is sold in powder form. However, although it is cheap, it is rarely used because it is less effective in decontamination when compared with sodium hypochlorite. Calcium hypochlorite is less liable to induce

tissue browning or injury, possibly due to the high concentration of Ca ions in solution (Sweet and Bolton, 1979).

Ethanol (70%) is the most widely used alcohol in sterilization of explants. Higher concentrations, like 96%, dehydrate plant tissues (Pierik, 1987). However, it is rare that an explant material can be successfully disinfected using ethanol alone.

2.5.2 Methods of effective use of sterilants and exposure time

A sterilant must make good contact with the entire surface of the explant. Penetration is considerably assisted by a short pre-immersion into 70% ethanol or by adding a non-phytotoxic wetting agent to the sterilant solution (George, 1993). Detergents used as wetting agents include Teepol® or Lisapol F® at 0,05-0.1%, Tween 20® or Tween 80® at 0,01- 0,05%, and Alconox® at 0.2% (George, 1993).

Both concentration and duration of exposure to disinfectants are important. Should these parameters be too high in terms of concentration or too long in terms of time, plant tissues are damaged, whereas too mild exposure does not eliminate microorganisms. A low concentration for a long period might not be as effective as a high concentration for a short interval (Gaiser *et al.*, 1981). Sterilization may be accomplished in a 5 minute treatment in 1% NaOCl in some plants, whereas in others 30 minutes serves as a benchmark (Pierik, 1987).

Lengthy sterilization with NaOCl can result in detrimental effects on the explant, thus, the correct timing and concentration for bleach should be decided at *ad hoc* basis for each plant species.

In early spring, buds from field-grown *Syringa* and some other woody ornamental shrubs were susceptible to NaOCl, with an exposure of one minute in a dilute solution causing 98% mortality (Einset and Alexander, 1985). Explants sampled later during the same season were more tolerant to NaOCl exposure, but then the explants were already highly contaminated with microbes and too old for successful culturing.

2.5.3 Reducing surface contamination

Field grown plants are generally heavily infected with various microbes (Dixon, 1986). However, after flushing the level of contamination changes with time within a given season (Einset and Alexander, 1985; Litz and Conover, 1977). Shoots of most field-grown plants are less contaminated during active growing stages.

High percentages of media infection occur when using materials from the field. Thus, it is necessary to use materials that were raised in the greenhouse, laboratory or growth room. In these enclosures, fungal and bacterial spores can be easily controlled (Dixon, 1986).

Plants should be potted in clean, sterilized containers using pasteurized potting mixture. The humidity should be kept as low as possible. Fungal and bacterial infections are more likely to occur at high humidity. Some researchers suggest that plants be raised in cool temperature and low relative humidity in order to suppress growth of microbes (Knauss and Knauss, 1980). When shoot or leaf material is to be used for explant, it is important that wetting of the upper part of the stock plant through splashing be avoided (Romberger *et al.*, 1970).

2.6 Choice of an appropriate medium

Media and methods that have been successful on genera of the envisaged plant species are suggested as a starting point for culturing an untested plant species (George *et al.*, 1988). In the absence of records for a given genus, those appropriate for members of the family could be evaluated. In case there are no records of family level, the next approach is to compare several well-known basal salt formulations. The most commonly used basal salt mixtures of Murashige and Skoog (MS) at half and full strength, could be compared with those of one or two other basal salt mixtures like Gamborg's B-5, McCown's woody plant and Nitsch and Nitsch basal salt mixtures. At this stage of testing, it would be advisable to use identical micronutrient and vitamin mixtures with sucrose concentration held constant at 2 or 3% (George *et al.*, 1988).

The MS media had successfully been used for shoot growth and shoot proliferation in several genera of woody fruit trees, such as *Citrus spp., Prunus spp., Pistachio* spp. and *Malus* spp. In some plants, it could be advantageous to increase or decrease the level of MS salts, keeping the ratio of inorganic ions constant (Whiteley and Abbott, 1977).

2.7 Growth substances and growth regulators in cultures

Plant growth substances (phyto-hormones) are chemicals or compounds occurring naturally within one plant tissue and transported to have a regulatory role in growth and development of different plant tissues. These compounds are generally active at low concentrations. Synthetic chemicals that have similar physiological activities like phyto-hormones are called plant regulators (Hartmann *et al.*, 1981). Recognized classes of plant growth regulators are auxins, cytokinins, gibberellins, abscisic acid and ethylene.

2.7.1 Action of plant hormones and growth regulators in cultures

Auxins and cytokinins are the most important plant hormones in regulating growth and morphogenesis in plant tissue and organ cultures. Generally auxins are produced in shoots and regulate root growth, whereas cytokinins are produced in roots and regulate shoot growth.

2.7.1.1 Auxins as growth regulators

Auxins are compounds capable of controlling various distinctive processes such as cell growth and cell elongation. They are also capable of initiating cell division and are involved in the development of meristems giving rise to either unorganized tissues (callus) or defined organs for the maintenance of apical dominance. Black *et al.* (1986) reported that 3-indole-acetic acid (IAA) was the most commonly detected natural auxin. It is usually used in combination with cytokinins [especially benzyladenine (BA) and kinetin] in explant initiation and shoot multiplication of cultures. Synthetically prepared IAA, when used as a source of auxins in plant tissue culture media, tends to be denatured and rapidly metabolized within the plant tissues. The attribute is useful because callus is induced by IAA and BA induces immediate shoot proliferation when the IAA concentration diminishes (George, 1993).

In some cases it may be necessary to use one of the many chemical analogues of IAA, since they have similar biological properties. Commercially available analogues of IAA include 2,4-dichlorophenoxyacetic acid (2,4-D), 3-Indolebutyric acid (IBA) and Naphthalene acetic acid (NAA). Together with cytokinins, 2,4-D is primarily used for callus induction. Naphthalene acetic acid (NAA) and 3-Indolebutyric acid (IBA) could replace IAA when morphogenesis is required for shoot tip cultures (Chaturvedi *et al.*, 1987).

2.7.1.2 Cytokinins as growth regulators

Cytokinins are often used to stimulate shoot growth and development. Kinetin, Benzyladenine (BA) and N^6-(*y, y*-dimethylallylamino) purine (2-iP) are the most commonly used cytokinins in cultures. The effect of these regulators is noticeable in tissue culture when they are used together with auxins to stimulate cell division and to control morphogenesis (George, 1993). When added to shoot culture media, these compounds overcome dominance and release lateral buds from dormancy. In certain cases, BA induces shoot proliferation in *Catalpa ovata* (a medicinal woody plant in Asia) with no addition of auxin to the medium (Lisowska and Wysokinska, 2000). Incorporation of IAA to the medium led to a decrease in shoot proliferation of *C. ovata*, whereas shoot elongation was better compared to those obtained from the medium containing only BA.

Kinetin and BA are usually used (alone or in combination) for axillary shoot sprouting. Superiority of BA and kinetin combination has been found in micro propagation of most woody perennials, e.g. cashew nut (Das *et al.*, 1996; Komalavalli and Rao, 1997). A combination of BA and Kinetin in *Gymnema sylvestre* (a multipurpose perennial woody medicinal plant) in the culture medium promoted shoot sprouting frequency and multiple shoot induction. Zeatin and N^6-(*y, y*-dimethylallylamino) purine (2-iP) are natural cytokinins that are structurally related to kinetin (Hecht, 1980). Commercial laboratories do not use natural cytokinin such as 2-iP and zeatin routinely because of their cost. The most

commonly used cytokinins in micropropagation are kinetin and benzyladenine (Kn).

2.7.1.3 Auxin and cytokinin actions in shoot cultures

In shoot tip cultures, growth regulators required depend to a large extent on the cultural stage and the envisaged type of plant growth. Auxins such as IAA, IBA and NAA are most frequently used, whereas 2,4-D is avoided because it induces unwanted callus growth (Hammerschlag, 1988). Benzyladenine has been used for a high proportion of shoot cultures along with kinetin and 2-iP.

Cytokinins are added to the medium to encourage the growth of axillary buds, and reduce apical dominance in shoot cultures. The effectiveness of cytokinins for promoting shoot proliferation in newly initiated cultures, particularly those of woody perennials, can depend on the time of the year and the stage of growth of the shoots from which explants are excised (Brand, 1993).

In some woody species, axillary shoot proliferation is promoted by the presence of auxins together with cytokinins. Sha Valli Khan *et al.* (1997) reported that a combination of cytokinins and auxins produced more shoots from *Syzygium alternifolium* (medicinal fruit tree of South India) explants than on the medium containing only cytokinins. The highest multiplication rate was achieved with nodal bud explants in the presence of 17.7 μM BA and 2.6 μM NAA.

In rooting of cultures, root formation is generally inhibited by the high concentration of cytokinins used to induce shoot multiplication or shoot formation *in-vitro*. Since cytokinins tend to inhibit root formation, it is necessary to transfer shoot clumps to another medium containing only auxins. Omitting cytokinins from the medium causes shoots of some plants (e.g. Fiddle-back fig, *Ficus lyrata*) to root spontaneously (Jona and Gribaudo, 1987). Once shoots are rooted, axillary shoot formation is usually inhibited.

Chapter 3

IN-VITRO PROPAGATION OF MMUPUDU

Currently, there is no evidence of micropropagation of any of the six species of the genus *Mimusop*, or within the Sapotaceae family. Thus, the literature reviewed gave a proper guidance on how to approach micropropagation of Mmupudu because most of the reviewed literature was mainly on woody tree *spp*.

3.1 Materials and Methods

The study was initiated at the University of the North on 01 September 1999 to test the contamination level of explants from the field and to evaluate various sterilants. Explants were obtained from forest-growing Mmupudu trees on the Sterkpoort mountain range, at Chuenespoort, 40-km south of Pietersburg, and at Sekgosese, which is in the Lowveld. Young branches with actively growing shoots were collected from mature Mmupudu trees and immersed in cool distilled water to minimize wilting. Samples were transported to the laboratory of the Department of Botany, University of the North. On arrival, 20-30 mm long shoots, each with two nodes, were excised from branches and leaves were removed prior to sterilization.

3.1.1 Sterilization of cultures and initiation

Pre-sterilization cleansing comprised placing explants for 2 hours under running tap water and then rinsing for 3 minutes in 60°C warm water. Prior to sterilization procedures, water was allowed to drain off from the shoots. Explants were disinfected for 10 minutes in 70% ethanol and then rinsed 3 times with sterile distilled water under a laminar flow bench. The explants were surface sterilized in 3% sodium hypochlorite solution plus 3 drops of a wetting agent (Tween-20®) for 10 minutes. Explants were again rinsed 3 times with sterile distilled water. Both ends of the explants were exposed to the sterilant, trimmed and vertically placed in glass culture vessels containing the MS basal media (Murashige and Skoog, 1962).

3.1.2 Composition of growing medium and culture conditions

The basal culture medium used was a modified MS medium with macro and microelements at half strength. The medium was supplemented with 30 g sucrose and 50 mg myo-inositol per liter distilled water. Because this was a trial to assess contamination, hormones were not added. The pH was adjusted to 5.8 and the medium was solidified with 7 g agar per liter distilled water prior to autoclaving at 121°C for 20 minutes. All cultures were maintained in a growth room at 26 ± 2 °C with a 16/8 hour light/dark photoperiod (35-90 $\mu E.m^{-2}.s^{-1}$) for

growth. Cool white fluorescent tubes provided light for growth. Relative humidity of the growth room was maintained at 55-60%.

Fungal contamination was noted from cultures within 2 to 4 days. Thus, pre-treatments became necessary to circumvent the problem. To evaluate the effect of various pre-treatments on explant contamination, different sterilization procedures were adopted.

First trial: Explants were soaked for 10 minutes in 70% ethanol, rinsed 3 times in sterile distilled water, surface sterilized for 15 minutes in 3% NaOCl containing Tween 20® and then finally rinsed 3 times in distilled water.

Second trial: Explants were pre-sterilized for 5 minutes in 90% ethanol, rinsed 3 times in sterile deionised water, surface sterilized for 15 minutes in 5% NaOCl containing Tween 20® and finally rinsed 3 times in sterile deionised water.

Third trial: Explants were continuously agitated in Blue solution (ammonium based disinfectant) for 10 min, surface sterilized for 5 minutes in 50 ml *Domestos*® (commercial bleach containing 5% NaOCl as an active ingredient) containing Tween-20® and then finally rinsed 3 times in distilled water.

Explants were transferred from every trial into fresh media every five days. Observations on contamination and percent survival of explants were recorded daily.

Explant establishment was difficult due to continuous contamination of explants obtained from mature field growing Mmupudu fruit trees and also because of electricity failures at the University. Because of these factors, experiments were moved to a private company named African Biotechnologies (Pty) Ltd, in Tzaneen, 70 km from the University of the North, on 12 April 2000.

3.2 Experiments at African Biotechnologies Laboratory

Eight young Mmupudu plants sexually propagated by seeds, in 5-L plastic bags, were purchased from 'Kannie Dood' nursery in Potgietersrus. The 75-cm tall plants were maintained in the greenhouse and pre-treated every other day with Sporegon sprays (a prochloraz manganese chloride based fungicide) for one week before sampling explants for culturing.

3.2.1 Sterilization and initiation of cultures

Leafy shoots developing from plants were used as explants. After removing leaves, shoots were trimmed into 20-30 mm long sections each consisting of two nodes. Further elimination of diseases and microorganisms was done by

washing explants for 10 minutes in *Bronocide*® (a glutaraldehyde based disinfectant). The explants were surface sterilized for 5 minutes in 50% *Domestos*® solution plus 3 drops of Tween 20®, with continuous agitation under the laminar flow bench. Thereafter, explants were rinsed 3 times with sterile distilled water. Before culturing, bases of explants exposed to the sterilant were trimmed back and cut ends were vertically inserted inside the magenta GA7 vessels containing the initiation medium.

3.2.2 Composition of growing medium and culture conditions

The basal culture medium used was a modified MS basal salt mixture with macro and microelements at full and half strength (Murashige and Skoog, 1962). The medium was supplemented with 30-g/L sucrose, 4.44 and 8.87 μM BA, 0.57 μM IAA and 50-mg/L myo-inositol. In each vessel, 0.682 g $CaCl_2$ + 0.624 g $Ca(NO_3)_2$ + 0.240 g $Mg(SO_4)_2$ per liter of distilled water were added to the growing medium of each treatment in order to alleviate shoo- tip necrosis. The pH was adjusted to 5.8 with 1M KOH or 1N HCl and thereafter, the medium was mixed with 6.75 g agar per liter distilled water and autoclaved for 15 minutes at 121°C. Thereafter, the media were poured into magenta GA7 vessels under a laminar flow bench. All cultures were maintained at 26° ± 2 °C in growth room with a 16/8 hour light/dark photoperiod (33-90 μE. m^{-2} .s^{-1}). Cool white fluorescent tubes provided light for growth. Relative humidity of the growth room was maintained at 55-60%. Cultures were checked for contamination daily. At harvest, 50 days after

initiating cultures, data collected included (i) the number of axillary shoots (the number of shoots that were produced on each axil of the explant), (ii) shoot length (the length of the axillary shoot that came out of the explant) and (iii) explant height.

3.2.3 Shoot proliferation, shoot-tip necrosis and experimental design

Experiment 1: MS with Benzyladenine (BA)

Benzyladenine (BA) was evaluated for shoot proliferation at 0.00, 6.60, 8.87, 11.07 and 13.32 μM and necrosis which was observed during shoot proliferation. The five treatments were arranged in the growth room in a randomized complete block design (RCBD) with 5 replicates each. Each culture vessel contained four explants.

Experiment 2: Kinetin and BA

Kinetin was tested for shoot proliferation at 0.00, 4.65, 9.29, 13.95 and 18.60 μM, with BA added in each level at 8.87μM. Treatments were laid out in the growth room in a RCBD with 5 replicates.

Experiment 3: MS with N^6-(*y, y*-dimethylallylamino) purine (2-iP)

N^6-(*y, y*-dimethylallylamino) purine was tested for shoot proliferation at 0.00, 4.92, 9.84, 14.76 and 19.68 μM. Also, treatments were laid out in the growth room in a RCBD with 5 replicates.

Experiment 4: Rooting proliferated shoots

Shoots with at least three expanded leaves that regenerated from cultures on the multiplication medium were used for the rooting experiments. The composition of the medium contained half-strength MS with 50 mg myo-inositol, 30 g sucrose, 6.7 g agar, 2 g charcoal per liter distilled water. Treatments were 0 and 9.80 μM IBA, arranged in the growth room in a completely randomized design with 120 replications.

At harvest, 50 days after initiating the cultures in all trials, rooted shoots were removed from the culture vessels and roots were washed in sterile distilled water to remove traces of agar. Data collected were (i) the number of roots per explant and (ii) the length of roots per explant.

Experiment 5: Weaning of plantlets

Plantlets were transplanted into 240 plug trays with each hole having a 3-cm diameter and 6-cm depth. A total of 150 plantlets were planted into each tray. The growing medium used in trays was peatmoss, supplemented with a 100-day slow release Horticote 2:1:2 fertilizer at 500 g/cm^3 of peatmoss. Lime was added to the peatmoss at 3 kg/cm^3 to adjust the pH to 6.0 and the cation exchange capacity to 2.8. Plantlets were first hardened in the greenhouse and then in the nursery, with the hardening rate computed for each location.

3.2.4 Data analysis

Data were subjected to analysis of variance in Experiment 1, Experiment 2 and Experiment 3 using Statistix program. Treatment means were separated using the least significant difference (LSD) test when F-values were significant ($P \leq 0.05$). In Experiment 4, since the untreated controls did not generate roots, a one-sample t-test was used; whereas in Experiment 5 hardening percentages were computed. In the first four experiments, unless otherwise stated, treatment means were significant and non-significant at $P \leq 0.01$ and $P \geq 0.05$, respectively.

4. Results

Preliminary trials: Explants establishment *in-vitro* was difficult when using explants that were collected from field-growing mature Mmupudu trees due to the high rate of contamination. The major contaminants were fungi and bacteria of different species.

Healthy shoots were obtained from 20-30-mm long shoot tips excised from actively growing shoots of Mmupudu plantlets that were maintained in the greenhouse. These shoot tips were successfully surface sterilized with *Bronocide*® and *Domestos*® to remove the bacteria and fungal contaminants.

Continuous treatment of plants in the greenhouse with S*poregon*® (a fungicidal spray) reduced fungal infections on plants.

Within two weeks of culture, most explants cultured in half-strength MS basal salt medium sprouted and produced new shoots, whereas those in full-strength turned brown and died within two weeks. From these results, further experiments were carried out using only half-strength MS growing medium (Figure 1a).

Experiment 1: MS with Benzyladenine (BA)

In a BA trial, the highest numbers of shoots per explant were at a concentration of 8.87 μM BA, which produced an average of 1.70 shoots (Table 1). The lowest number of shoots occurred at the concentration of 13.32 μM BA and untreated control had no shoots. Benzyladenine treatment contributed 37% to the total treatment variation in the number of shoots produced per explant. Blocks had no effect on shoot number variation.

Shoot lengths at BA concentrations of 6.60, 8.87 and 11.07 μM were not different ($P \leq 0.05$), but were higher when compared with those of the control and 13.32 μM BA. Benzyladenine contributed 8 % ($P \leq 0.1$) to the total treatment variation in length of shoots produced, with blocks contributing 18.9% to the total treatment variation in shoot length.

The explants elongated best at BA concentration of 8.87 and 11.07 μM, whereas those at 6.60 and 13.32 were not significantly different ($P \leq 0.05$). Benzyladenine (BA) contributed 15 % to the total treatment variation in the height of explants.

Shoot-tip necrosis that appeared to be a problem during shoot proliferation was reduced by the addition of Ca and Mg ions supplements that was added to the medium with Benzyladenine (BA).

Table 1. Effect of BA on shoot proliferation of Mmupudu after 16 weeks in culture.

Treatment BA (μM)	Shoot number per explant	Shoot-tip necrosis	Shoot Length (mm) per explant	Height (mm) Per explant
0.00	0.00 d	0.00 c	0.00 d	0.00 c
6.60	1.05 b	1.45 ab	14.4 ab	25.1 b
8.87	1.70 a	1.25 b	15.7 a	27.4 a
11.07	0.85 bc	1.85 a	14.1 ab	27.8 a
13.32	0.70 c	1.90 a	11.0 c	26.1 b
% Trt. V.	36.9	12%	7.8	14.5
P value	0.01**	0.01**	0.09*	0.01**
MSE	0.15	0.23	0.21	0.07
CV	0.30	0.46	0.43	0.14

Means with the same letter were not different ($P \leq 0.05$) according to LSD test.

*; *** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

% Total treatment variation to the observed mean.

Experiment 2: Kinetin and BA at 8.87μM

Cultures initiated in the MS medium supplemented with 8.87 μM BA and 1.14 μM IAA showed apical buds sprouting within two weeks of culturing. About 80-90% of the plants produced new shoots. At a constant level of BA, the highest number

of new shoots per explant was observed when kinetin concentration was 9.29 μM (Figure 1b). The second best kinetin concentration was at 4.65 μM. At the highest kinetin concentration of (18.6 μM) shoot proliferation was poor, with most leaves undergoing chlorosis (Table 2). Kinetin plus BA contributed 56% to the total treatment variation in the number of shoots produced per explant. Blocking contributed 10% to the total treatment variation in the number of shoots per explant.

The longest shoots occurred at 4.65 and 9.29 μM kinetin, whereas shoot length was suppressed by higher concentrations. Benzyladenine (BA) plus kinetin contributed 9% ($P \leq 0.05$) to the total treatment variation in shoot length of Mmupudu, whereas blocking contributed 15% to the total treatment variation in shoot length. The control treatment did not produce shoots because no hormones were added to the medium.

Table 2. Effect of Benzyladenine (BA) and Kinetin (KN) on shoot multiplication of Mmupudu after 16 weeks in culture.

Treatment		Shoot number per	Shoot length (mm) per
BA (μM)	KN (μM)	Explant	Explant
0.00	0.00	0.65 d	10.0 c
8.87	4.65	1.45 b	15.3 a
8.87	9.29	2.05 a	17.4 a
8.87	13.95	1.10 c	12.1 b
8.87	18.60	1.00 c	12.9 b
% Trt. V.		55.8	9.18
P value		0.01**	0.03*
MSE		0.15	0.19
CV		0.29	0.38

Means with the same letter were not different ($P \leq 0.05$) according to LSD test.

*; **Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

% Total treatment variation to the observed mean variation.

Experiment 3: MS with N^6-(*y, y*-dimethylallylamino) purine (2-iP)

Shoot proliferation did not occur when N^6-(*y, y*-dimethylallylamino) purine (2-iP) was used in the shoot proliferation medium (Figure 1c).

Experiment 4. Rooting proliferated shoots

Excised shoots rooted readily within 8 weeks of transfer to half-strength MS supplemented with 9.40 μM IBA and 2 g activated charcoal per 1L of sterile distilled water. Within eight weeks of initiating the rooting trial, about 80-90% of the explants produced 2 to 3 white adventitious roots on the basal end (Figure 1d). The number of roots and root lengths were significantly different ($P \leq 0.01$) from those in untreated controls (Table 3).

Table 3. Effects of IBA on root proliferation of Mmupudu 8 weeks after culturing.

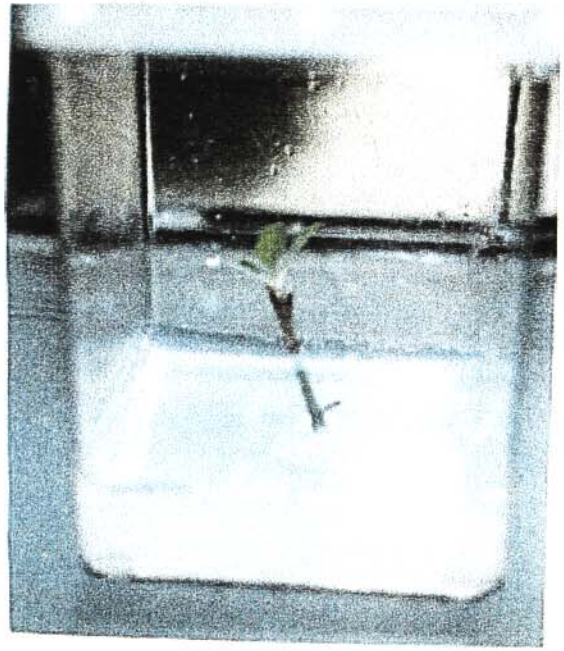
	Experiment 1		Experiment 2	
Statistics	Root numbers	Root length (mm)	Root numbers	Root length (mm)
Mean	3.225	1.275	1.275	2.688
Std error	0.336	0.139	0.139	0.271
Low 95% CI	2.557	0.999	0.999	2.148
Up 95% CI	3.893	1.552	1.552	3.227
T	9.61	9.18	9.18	9.92
Df	79	79	79	79
P value	0.001***	0.001***	0.001***	0.001***

One-sample t-test for root proliferation from explants.

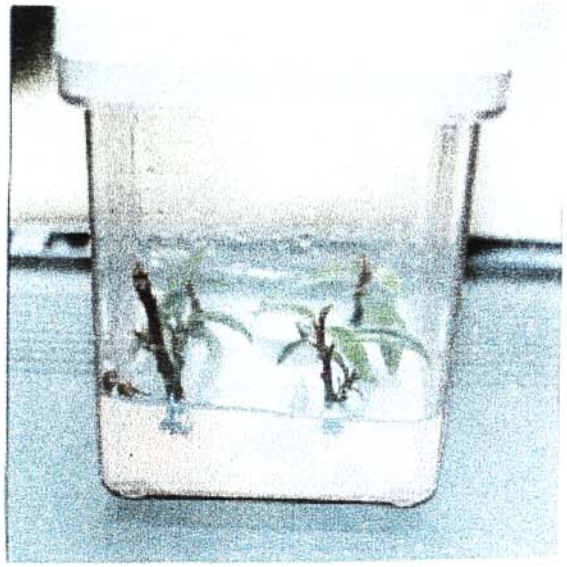
*** Significant at $P \leq 0.01$

FIGURE 1 (a) New shoots sprouting from Mmupudu explant on the initiation medium.
(b) Shoots proliferation on the multiplication medium that was supplemented with 8.87 μM Benzyladenine in combination with 9.29 μM Kinetin.
(c) Poor shoot proliferation on the multiplication medium supplemented with N^6- (y' y-dinethylallylamino) purine.
(d) A rooted shoot of Mmupudu cultured on half strength MS containing 9.40 μM 3-Indolebutyric acid.

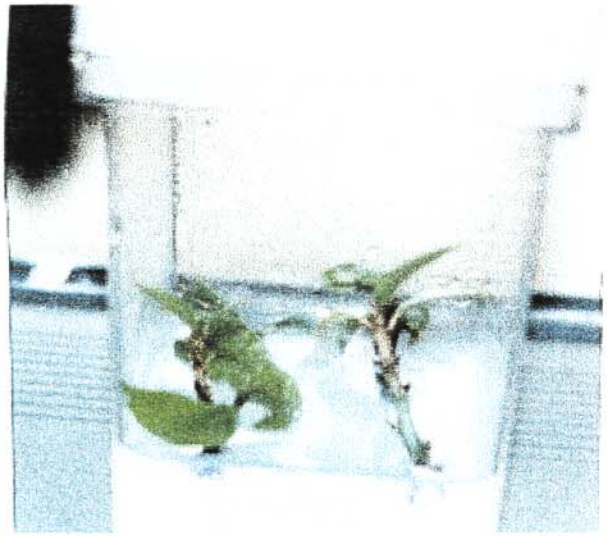
A



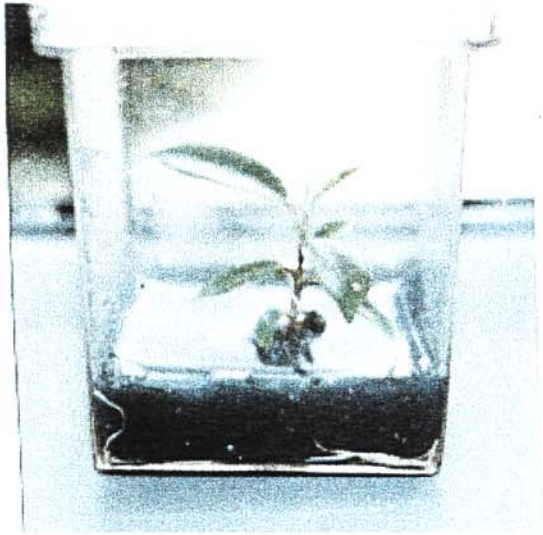
B



C



D



Experiment 5. Hardening of rooted plants from the laboratory

Rooted plantlets with fully expanded leaves and well-developed roots from the laboratory were transferred into trays containing peat moss. The plants were acclimatized in the greenhouse (Figure 2). Normal growth of plants with new shoots was observed within 4 weeks after hardening.

Four-months later, plants had normal leaves and were phenotypically similar to the mother plant, and were transplanted into 2-L plastic bags containing a mixture of sterilized peat moss (Figure 3). Plants developed adventitious roots, which grew faster than shoots. Success rate during hardening was 60 % in trays and 50% in bags.

Figure 2: Acclimatized plantlets of Mmupudu 4 weeks after planting in trays in the greenhouse (above).

Figure 3: Normal growth of micropropagated plant 4 months after transplanting in 2 litre black nursery bags containing soil.

5. Discussion

In preliminary trials, *in-vitro* establishment of Mmupudu explants was unsuccessful due to the high rate of contamination. This observation confirmed previous reports that demonstrated that field-growing plants were not suitable sources for explanting. Plants growing in the external environment are always exposed to contamination by fungi, bacteria and other microorganisms. The spore counts on field-growing plants are always higher than for those under greenhouses, growth chambers or laboratories. Similar results were observed on *in-vitro* propagation of *Pistachio* where high contamination of field grown trees precluded the use of these materials as sources for *in-vitro* explants (Barghchi, 1982; Bustamante-Garcia, 1984; Abousalim, 1990). Raising greenhouse plants as sources for explant materials is essential to the success of *in-vitro* propagation. Generally, it is recommended that foundation plants for tissue culture work should be initiated from clean and disease-free plant materials and maintained as such until use (George, 1993). Greenhouse foundation block enhanced the establishment of *in-vitro* propagation of Mmupudu. Most Mmupudu explants from the greenhouse were clean whenever they were initiated *in-vitro*.

The use of explant material from younger Mmupudu plants was advantageous because when cultures were introduced to the nutrient medium, there were no phenolic exudates into the media. In cases where explants were from mature trees, media stained as a result of exudates. This supported the view that young

juvenile plant tissues were less prone to browning on excision than older tissues because older tissues exuded more phenols into the growing medium (Muhitch and Fletcher, 1984).

The successful *in-vitro* propagation of Mmupudu using greenhouse and young budwood create some problems. Amongst other reasons, micropropagation of Mmupudu was intended to produce clonal rootstocks, from a parent with known characteristics. Thus, the observation that *in-vitro* propagation was successful using greenhouse and young materials suggested that asexual propagation should be evaluated so that the resultant plants can be decontaminated over time in the greenhouse and serve as the foundation plants for *in-vitro* propagation.

Woody plants are sensitive to full strength media due to ion toxicity, thus it is advisable to use half-strength basal salt mixtures for woody plants (George *et al.*, 1988). In this study, tissue browning occurred as a result of using full-strength MS basal salt medium, which invariably led to the mortality of explants within 2 weeks of culturing. Half-strength basal salt mixtures and BA plus IAA were the most effective for the establishment of Mmupudu. Generally, a combination of auxins and cytokinins in the growing medium releases buds from dormancy, thus, enhancing leaf formation (George, 1993).

Axillary shoot sprouting was initiated in all the cytokinin treatments tested. Results showed that addition of BA and kinetin to half-strength MS basal salt medium was more effective in shoot proliferation than 2-iP and BA alone. Cytokinin 2-iP induced low proliferation of shoots when compared with other cytokinins. Onay (2000) reported the same responses to 2-iP in *in-vitro* propagation of *Pistachio*. Also, Onay (2000) observed low multiplication frequencies when 2-iP was used as sole cytokinin source on *Pistachio* when compared with BA.

In Mmupudu, (BA) alone at different concentrations induced lower shoot proliferation when compared with BA plus kinetin. Komalavalli and Rao (2000) reported similar findings on the use of BA plus kinetin on *G. sylvestre* explants. In *G. sylvestre*, shoot proliferation was optimal when a combination of 6.6 µM BA and 2.33 µM kinetin were used. Superiority of BA in combination with kinetin had been reported on micropropagation of woody plants such as cashew nut (Das *et al.*, 1996) and *G. elegans* (Komalavalli and Rao, 1997).

Blocking effects on shoot numbers per explant during proliferation might be due to light intensity since the effects were consistently stronger in blocks adjacent to the light source. The role of light intensity on elongation of Mmupudu is not clear, because shortage or deprivation of light during the growing stages of plants is the one factor that is known to enhance shoot elongation (Hartmann *et al.*, 1981). In nature, Mmupudu inhabits shaded areas (Venter and Venter, 1996), thus, it is not

known whether the response of this plant to high light intensity is similar to that of light-loving plants.

Shoot-tip necrosis of Mmupudu, followed by leaf senescence in cultures, was successfully corrected using various sources of Ca ions. Other studies showed that shortage of Ca was associated with shoot tip necrosis in *Betula*, *Populus* and other woody plants (George, 1993). In this study, addition of calcium nitrate and calcium chloride to the medium in combination with magnesium chloride reduced necrosis. Selection of 3-Indole-butyric acid (IBA) for rooting of Mmupudu *explants* was based on previous experiments which reported the use of IBA on rooting of woody plants (Onay 2000; Komalivalli and Rao, 2000). The stimulatory effect of IBA on rooting was also found in *Syzygium alternifolium* (Sha valli Khan *et al.*, 1997). Similar rooting responses using IBA as the rooting hormone for woody plants had been reported for *Pistachio* (Onay, 20000), *Catalpa ovata* (Manojlovic *et al.*, 1994) and *G. sylvestre* (Komalavalli and Rao, 1997).

Greenhouse and nursery weaning had success rates of 60% and 50%, respectively. These rates are more or less similar to the germination rate of 60-80% in Mmupudu seeds (Venter and Venter, 1996). The reasons for these low hardening rates are not yet clear.

3.6 CONCLUSIONS

Plants raised under greenhouse conditions served as the best source of explant material for the successful *in-vitro* propagation of Mmupudu because fungal and bacterial spores inoculum levels are low and can be easily controlled using various sprays. Shoot tips obtained from juvenile plants were better than those from mature plants because of low phenols in juvenile plants. Also, young shoots grew faster and were more prolific than mature plant parts *in-vitro*. Murashige and Skoog basal salt medium at half-strength was suitable for Mmupudu propagation. Benzylaminopurine (BA) at 8.87 μM and kinetin at 9.29 μM were the best for shoot proliferation of Mmupudu, whereas the best rooting hormone was 3-indole-butyric acid at 9.40 μM. Shoot proliferation, root initiation and establishment in the greenhouse occurred in less than 31 weeks. Thus, should hardening rates be improved, *in-vitro* propagation of Mmupudu have the potential of serving as a technique for mass propagation of this fruit tree for small-holder farming systems.

REFERENCES

ABOUSALIM A. 1990. Micropropagation and micrografting of Pistachio (*P.vera* L. and *Pistacia atlantica* Desf.). Ph.D. Thesis, Wye College, University of London, UK.

BARGHCHI M. 1982. *In-vitro* propagation of *Pistacia* species. Ph.D. Thesis, Nottingham University, UK.

BARGHCHI M. 1986. *In-vitro* micropropagation of *Pistacia* rootstocks. Comb. Proc. Int. Plant Prop. Soc. **35**, 331-333.

BLACK R.C., KULECK G.A. & BINNS A.N. 1986. The initiation of auxin autonomy in tissue from tobacco plants carrying the auxin biosynthesizing genes from the T-DNA of *Agrobacterium tumefasciens*. Plant Physiol. **80**, 145-151.

BRAND H.M. 1993. Initiating cultures of *Halesia* and *Malus*: influence of flushing stage and benzyladenine. Plant Cell Tiss. Org. Cult. **33**, 129-132.

BUSTAMANTE-GARCIA M.A. 1984. Micropropagation and rejuvenation of *Pistacia* species and the mechanism by which light influences root initiation. Ph.D. Thesis, University of California, Davis, US.

CHATURVEDI H.C., SHARMA A.K. & PRASAD R.N. 1987.Shoot apex culture of *Bougainvillea glabra* 'Magnifica'. HortScience **13**, 36.

CHALUPA V. 1996. *Fagus sylvatica* L. (European Beech). Int: Bajaj YPS (ed). Biotechnology in Agriculture and Forestry, vol 35. Trees IV (pp 138-154). Springer-Verlag: Berlin.

DAS S., TIMIR B.J. & SUMUTA J. 1996. *In-vitro* propagation of cashew nut. Plant Cell Rep. **15**, 615-619.

DAVIES M.E. & DALE M.M. 1979. Factors affecting *in-vitro* shoot regeneration on leaf discs of *Solanum laciniatum*. Z. Pflanzenphysiol. **92**, 51-60.

DEBERGH P.C. 1988. Micropropagation of woody species-state of the art on *in-vitro* aspects. Acta Hort. **227**, 287-295.

DE FOSSARD R.A. & BOURNE R.A. 1976. Vegetative propagation of *Eucalyptus ficifolia* F. Muell by nodal culture *in-vitro*. Comb. Proc. Int. Plant Prop. Soc. **26** ,373-378.

DIXON R.A. 1986. Plant Cell Culture. A practical approach. Egham Hill, Egham. Surrey TW 20 EX, UK.IRL Press: Oxford Washington DC.

DYCHDALA G.R. 1977. pp. 167-195 in Block S.S. (ed) Disinfection, sterilization and preservation. Lea and Febiger: Philadelphia.

EINSET J.W. & ALEXANDER J.H. 1985 Multiplication of *Syringa* species and cultivars in tissue culture. Comb. Proc. Int. Plant Prop. Soc. **34**, 628-636.

GAISER M.S., LAZARTE J.E & BROWN O.R. 1981. *In-vitro* propagation of *Epiphyllum chrysocardium*. HortScience **16**, 425 (Abst. 194).

GARTON S. & MOSES M.S. 1986. Production of native plants in tissue culture. Comb. Proc. Int. Plant Prop. Soc. **35**, 306-315.

GEORGE E.F. 1993. Plant propagation by tissue culture. Part I. The technology. 2nd ed. Exegetics: Edington.

GEORGE E.F., PUTTOCK D.J.M. & GEORGE H.J. 1988. Plant culture media. Exegetics: Westbury.

HARTMANN H.T., FLOCKER W.J. & KOFRANEK A.M. 1981. Growth, development and utilization of cultivated plants. Plant Science. 2nd ed. Prentice-Hall: London.

HECHT S.M. 1980. Probing the cytokinin receptor site(s). pp. 144-160. In Skoog F. (ed.). Tissue culture. Wiley: New York.

HAMMERSCHLAG F.A. 1988. Callus and root formation from leaves of mature peach plants propagated *in-vitro*. HortScience **23**,756.

HUTCHINSON J.F. 1982. *In-vitro* propagation of apple using organ culture. pp. 729-730 in *Fujiwara* (ed.) 1982 (q.v.).

JONA R. & GRIBAUDO I. 1987. Adventitious bud formation from leaf explants of *Ficus lyrata*. HortScience **22**, 651-653.

KNAUSS J.F. & KNAUSS M.E. 1980. Contamination in plant tissue cultures. Proc. Fla. State Hort. Soc. **92**, 341-343.

KOMALAVALLI N. & RAO M.V. 1997. *In-vitro* micropropagation of *Gymnema elegans* W and A, a rare medicinal plant. Indian J. Exp. Biol. **35**, 1088-1092.

KOMALAVALLI N. & RAO M.V. 2000. *In-vitro* micropropagation of *Gymnema sylvestre*-A multipurpose medicinal plant. India. Plant Cell Tiss. Org. Cult. **61**, 97-105.

LISOWSKA K. & WYSOKINSKA H. 2000. *In-vitro* propagation of *Catalpa ovata* G. Don. Poland. Plant Cell Tiss. Org. Cult. **60**, 171-176.

LITZ R.E. & CONOVER R.A. 1977. Tissue culture propagation of some foliage plants. Proc. Fla. State Hort. Soc. **90**, 301-303.

MANOJLOVIC M., VINTERHALTER B. & VINTERHALTER D. 1994. *In-vitro* propagation of *Catalpa ovata* G. DON and *Catalpa bignonioides* Walt. Glas. Inst. Bot. I Baste Univ. U Beogradu, **26-27**, 7-14.

MASHELA P.W. 1992. Interactions of citrus rootstocks, *Tylenchulus semipenetrans* and salinity. Ph.D. Thesis, University of Florida, Gainesville, US.

MASHELA P.W. & MOLLEL N. 2001. Farmer-identified indigenous fruit trees with suitable attributes for the semi-arid Northern Province of South Africa. S. Afr. J. Agric. Ext, 1-12.

McCOWN B.H. & SELLMER J.C. 1987. General media and vessels suitable for woody plant culture. pp. 4-16. In Bonga and Durzan (eds.). Plant tissue culture. Wiley: New York.

MEIER K. & REUTHER G. 1994. Factors controlling micro propagation of mature *Fagus sylvatica*. Plant Cell Tiss. Org. Cult. **39**, 231-238.

MUHITCH M.J. & FLETCHER J.S. 1984. Isolation and identification of the phenols of Paul's Scarlet rose stem derived suspension cultures. Plant Physiol. **75**, 572-575.

MURASHIGE T. & SKOOG F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. **15**, 473-479.

ONAY A. 2000. Micropropagation of Pistachio from mature trees. Plant Cell Tiss. Org. Cult. **60**, 159-162.

PALGRAVE K.C. 1977. Trees of Southern Africa. Struik: Cape Town.

PIERIK R.L.M. 1987. *In-vitro* culture of higher plants. Martinus Nijhoff: Boston.

ROMBERGER J.A., VARNELL R.J. & TABOR C.A. 1970. Culture of apical meristems and embryonic shoots of *Picea abies*-approach and techniques. U.S.D.A. Technical Bull. **1409**, 1-30.

SHA VALLI KHAN P.S., PRAKASH E. & RAO K.R. 1997. *In-vitro* micropropagation of an endemic fruit tree *Syzygium alternifolium* (Wight) walp. Plant Cell Rep: **16**,325-328.

SWEET H.C. & BOLTON W.E. 1979. The surface decontamination of seeds to produce axenic seedlings. Am. J. Bot. **66**, 692-698.

UNO S. & PREECE J.E. 1987. Micro- and cutting propagation of 'Crimson Pygmy' barberry. HortScience **22**, 488-491.

VENTER F. & VENTER J.A. 1996. Making the most of indigenous trees. Briza: Cape Town.

VIETEZ A.M., FERRO E.M. & BALLESTER A. 1993. Micropropagation of *Fagus Sylvatica* L. *In-vitro* Cell. Dev. Biol. **29**, 183-188.

WHITELEY E. & ABBOTT A.J. 1977. Microvegetative propagation of apples. Ann. Rep. Long Ashton Res. Sta. **1976**, 62-63.

ZIV M. & HALEVY A.H. 1983. Control of oxidative browning and *in vitro* propagation of *Strelitzia reginae*. HortScience **18**, 434-436.