MEDICINAL PROPERTIES AND *IN VITRO* RESPONSES OF *MAYTENUS SENEGALENSIS* (LAM.) EXELL

ESTHER NG'ENDO MATU

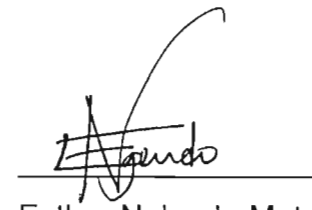
Submitted in fulfilment of the academic
requirements for the degree of
Doctor of Philosophy
in the
Research Centre for Plant Growth and Development,
School of Botany and Zoology,
University of Natal, Pietermaritzburg.

2003

DECLARATION

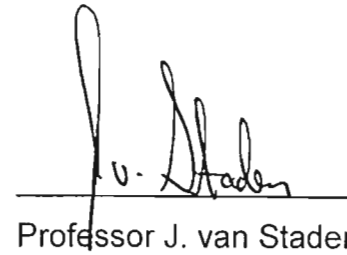
I hereby declare that the experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg from February 2000 to June 2003, under the supervision of Professor J. van Staden.

These studies are a result of my own investigations, except where acknowledgement of other work is specifically indicated in the text and have not been submitted in any other form to another University.



Esther Ng'endo Matu

I certify that the above statement is true.



Professor J. van Staden
(Supervisor)

PUBLICATIONS FROM THIS THESIS

MATU EN and VAN STADEN J (2003). Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. Journal of Ethnopharmacology **87**: 35-41.

PUBLICATIONS IN PREPARATION

MATU EN and VAN STADEN J. Micropropagation of *Maytenus senegalensis* (Lam.) Exell: A medicinal plant.

MATU EN and VAN STADEN J. The influence of some factors on adventitious root formation in *Maytenus senegalensis* (Lam.) Exell shoots.

MATU EN and VAN STADEN J. *In vitro* root cultures of *Maytenus senegalensis* (Lam.) Exell.

MATU EN and VAN STADEN J. Antibacterial activity of extracts prepared from *Maytenus senegalensis* (Lam.) Exell *in vitro* grown root cultures.

CONFERENCE CONTRIBUTIONS FROM THIS THESIS

MATU EN and VAN STADEN J (2001). Some medicinal properties of some Kenyan plants (Paper). Twenty-seventh South African Association of Botanists Congress, RAU, Johannesburg (South Africa).

MATU EN and VAN STADEN J (2001). Antibacterial and anti-inflammatory activities of some commonly used Kenyan medicinal plants (Paper). Ninth symposium of the Natural Products Research Network for Eastern and Central Africa (NAPRECA), Kenyatta International Conference Centre, Nairobi. (Kenya).

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my supervisor, Professor J. van Staden for patiently guiding me in the course of this study. Members of my research committee, Prof A. Jäger, Dr C. Fennel, Dr W. Stirk, Dr S. McCartan and Mrs K. Lindsey are sincerely thanked for their enthusiasm and helpful comments. I also wish to thank Dr E. Elgorashi for his dedicated help on phytochemical techniques.

The financial support from NRF (Pretoria, South Africa) is acknowledged. My employer, Kenya Medical Research Institute through the office of the Director is thanked for granting me study leave.

I am grateful to Mr Onesmus Mwangangi and Joshua Muasya for their help with the collection of plant material and subsequent authentication at the East-African Herbarium, Nairobi. Moses allowed me to collect parts of *Zanthoxylum usambarense* at the Rowallan Scouts camp forest, to whom am grateful.

To the members of staff at the Research Centre for Plant Growth and Development, particularly Mrs J. Magnussen, Mrs Lee Warren and Mr Martin Hampton, many thanks for joyfully offering assistance whenever I needed help.

To all my colleagues at the Research Centre for Plant Growth and Development, particularly Dr A. Vonjy, G. Stafford, G. Arthur and S. Sparg, many thanks for your ever available assistance. I owe a debt of gratitude to my friends in South Africa especially Dr (Rvd) and Mrs Walker, Dr and Mrs Njuhos family, Prof and Mrs Mbatis family and the Mureithiis for their support and encouragement during very difficulty moments of this research.

I would like to thank my dad, Mzee Matu and members of my family for their unfailing love and prayers. To my sister, Catherine, many thanks for even in your illness, your friendship and belief in me never wavered. To my niece Wiri, a special note of thanks for taking care of my daughter during my absence. Many thanks to Macharia for his friendship and moral support at all times. Lastly, but never the least, I would like to thank my daughter Sharon, for her immeasurable sacrifices, endurance and understanding during my endless circles of hope and despair. To God be all glory, great has been your faithfulness oh Lord!

ABSTRACT

Maytenus senegalensis (Lam.) Exell belongs to the Celastraceae family. Its extracts are widely used in traditional medicine for treatment of a variety of ailments. Probably due to its popularity in traditional medicine coupled with destruction of habitats, *M. senegalensis* populations are reported to be on the decline in east Africa. The objectives of this study were to screen extracts prepared from *M. senegalensis* for antibacterial and anti-inflammatory activities, isolate and identify some of the bioactive secondary metabolites, to develop a micropropagation protocol as a conservation option and to establish and evaluate the potentials of *in vitro* root cultures as alternative sources of antibacterial compounds.

In the first part of the study, extracts prepared from various parts of *M. senegalensis* alongside other plant species used for medicinal purposes in Kenya were screened for antibacterial and anti-inflammatory activities. Antibacterial activity was carried out using the disc-diffusion bioassay while anti-inflammatory activity was done using the cyclooxygenase (COX-1) assay. Antibacterial activity was only exhibited against Gram-positive bacterial strains. Aqueous and methanolic extracts prepared from *M senegalensis* root-bark and stem-bark exhibited high antibacterial activity while the leaf extracts had no activity. High antibacterial activity was also exhibited by extracts of *Conyza schimperiana*, *Galinsoga parviflora*, *Plectranthus barbatus*, *Spiranthes mauritianum*, *Zanthoxylum chalybeum* and *Z. usambarense*. In the COX-1 assay, methanolic extracts of all the plant species assayed, generally exhibited high anti-inflammatory activity compared to the aqueous and hexane extracts. The results obtained in this part of the study confirmed the therapeutic potency of the twelve plant species analysed thus providing a rationale for their use in traditional medicine. The use of *M. senegalensis* extracts in traditional treatment of infectious and inflammatory conditions was also confirmed. This formed a good basis for its selection as a good candidate warranting further pharmacological studies and conservation efforts.

In the second part of the study, an efficient micropropagation protocol was established using *in vitro* germinated 6-week-old seedlings as a source of explants. Shoot proliferation was only achieved using nodal explants. The presence of cytokinins was necessary for shoot induction and growth. While benzyl-6-aminopurine (BA) generally promoted the average number of shoots produced per explant, kinetin promoted the mean length of shoots. Inclusion of auxins (IAA and IBA) in the shoot induction and growth medium did not have significant effects on either the average number of roots produced per shoot or the mean length of shoots. Low levels of BA (0.5 or 1.0 mgl^{-1}) were optimal for shoot induction and growth. As a result, shoot multiplication was done on a medium supplemented with BA (0.5 mgl^{-1}) alone. Rooting was done in two stages. In the first stage, 8-week-old shoots (2-3 cm long) were pulse treated in the dark using ½ strength, MS (MURASHIGE and SKOOG 1962) liquid medium containing auxins and then transferred to a solid, hormone-free medium at 12/12 h photoperiod. The shoots pulse-treated with 25 mgl^{-1} IBA for 120 h produced the highest number of roots per shoot. IBA concentration and the period of pulse-treatment had significant effects on the average number of roots produced per shoot. The rooted plantlets were successfully acclimatized.

In the third part of the study, various factors were shown to have significant effects on adventitious root formation of *M. senegalensis in vitro* shoots. Rooting was totally inhibited in the absence of auxins while 28 °C was the optimal temperature for adventitious root formation. Rooting shoots on a liquid medium promoted the formation of long roots that had a thick tuft of root hairs. A significantly high number of roots were formed per shoot when 0.8% agar (Agar-Agar powder CP, Batch: 8522/151, Associated Chemical Enterprises c.c, South Africa) was incorporated into the medium but the roots were shorter than those formed on a liquid medium and lacked root hairs. The addition of activated charcoal to the root-growth medium significantly reduced the average number of roots per shoot. Low concentrations (1/4 or 1/2) of MS medium (MURASHIGE and SKOOG 1962) led to the production of a higher number of roots per shoot compared to higher concentrations (3/4 or full). A dark pre-treatment (120 h) or the presence of light had no significant effects on adventitious root formation.

In the fourth part of the study, *in vitro* root cultures of three *M. senegalensis* genotypes were successfully established. The presence of auxins was necessary for stimulation of root growth. NAA (0.5 mgl^{-1}) promoted the best response in terms of the fresh weight (FW) of the roots in all the genotypes. Root-tip necrosis was encountered in genotype B. The types of sugars and different concentrations of sucrose were found to significantly influence the growth of genotype A root cultures. Light (presence or absence) and the type of medium (agar-gelled or liquid) significantly influenced the growth of genotype A root cultures.

In the last part of the study, methanolic extracts prepared from 8-week-old *M. senegalensis in vitro* root cultures were screened for antibacterial activity. Antibacterial activity was only exhibited against Gram-positive bacterial strains. However, the extent of antibacterial activity was the same irrespective of the genotype. *M. senegalensis* genotype A root cultures grown on different MS media strengths (1/4, 1/2, 3/4 or full) also exhibited antibacterial activity to the same extent. Extracts prepared from roots cultured in the presence of high sucrose concentrations (100 mgl^{-1}) had no antibacterial activity. Roots cultured in the dark exhibited a slightly higher antibacterial activity against *Staphylococcus aureus* compared to the roots cultured in the presence of light.

TABLE OF CONTENTS

CHAPTER 1
INTRODUCTION

CHAPTER 2
LITERATURE REVIEW

CHAPTER 3
ANTIBACTERIAL AND ANTI-INFLAMMATORY ACTIVITIES OF SOME PLANTS USED FOR MEDICINAL PURPOSES IN KENYA

CHAPTER 4
MICROPROPAGATION OF *MAYTENUS SENEGALENSIS* (Lam.) Exell

CHAPTER 5

THE INFLUENCE OF SOME FACTORS ON ADVENTITIOUS ROOT FORMATION IN *MAYTENUS SENEGALENSIS*

CHAPTER 6

IN VITRO ROOT CULTURES OF *MAYTENUS SENEGALENSIS*

CHAPTER 7
ANTIBACTERIAL ACTIVITY OF EXTRACTS PREPARED FROM *MAYTENUS SENEGALENSIS IN VITRO* ROOT CULTURES

LIST OF ABBREVIATIONS

AC	Activated charcoal
ANOVA	Analysis of variance
BA	benzyl-6-aminopurine
COX	Cyclooxygenase
IAA	Indole-acetic acid
IBA	Indole-3-butyric acid
h	hours
Kinetin	N^6-furfuryadenine
Min	Minutes
MS	Murashige and Skoog (1962) medium
MH	Mueller-Hinton
NSAIDS	Non-steroidal anti-inflammatory drugs
NAA	α-Naphthaleneacetic acid
PGRs	Plant growth regulators
RPM	Revolutions per minute
SE	Standard Error
TLC	Thin layer chromatography
TMPs	Traditional Medical Practitioners
WHO	World Health Organization

LIST OF FIGURES

LIST OF PLATES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 THE GENUS *MAYTENUS*

Maytenus Molina is one of the chief genera of the plant family Celastraceae. It comprises of about 225 species (HEYWOOD 1978; MABBERLY 1987; WIELGORSKAYA 1995; EVANS 1996; HUTCHINGS *et al.* 1996), which are widespread in the tropical and sub-tropical regions of both the northern and southern hemispheres (ROBSON *et al.* 1994). In Africa, *Maytenus* species are widespread in the eastern parts of tropical Africa and southern Africa (ARCHER and JORDAAN 2000). Habitually, *Maytenus* species are either trees or shrubs (ROBSON *et al.* 1994).

The genus *Maytenus* is reportedly riddled with taxonomic difficulties (MARAIS 1960; ROGERS *et al.* 1999). Consequently, JORDAAN and VAN WYK (1999) recommended the reinstatement of the genus *Gymnosporia*, (Wight and Arn.) Hook, f. thus transferring about eighty species, which were initially in the genus *Maytenus*. However, there seems to be a lack of consensus at the international level since no such nomenclatural changes have been effected at the East-African Herbarium, Nairobi, Kenya or at the Royal Botanic Gardens, Kew, where the Flora of Tropical East Africa (FTEA) is compiled (HENKE BEENTJE, pers. com., March 2003). The name *Maytenus* is hence retained as far as this study is concerned. Nevertheless, some workers especially in the southern African countries have adopted the name *Gymnosporia* for the affected species including *M. senegalensis* (ARCHER and JORDAAN 2000; VAN WYK *et al.* 2000; ROODT 1998). In East Africa, the name *M. senegalensis* is retained with *G. senegalensis* being treated as the synonymn (DHARANI 2002).

1.2 MEDICINAL IMPORTANCE OF *MAYTENUS* SPECIES

1.2.1 Role in traditional medicine

Utilization of several *Maytenus* species in treating all manner of ailments in various parts of the world is widely documented in ethnomedical literature (Table 1.1). Their widespread occurrence in the tropical and sub-tropical regions of the world implies that they are found in regions with a long history of traditional medicine usage. In some instances, some species are reportedly used in treating the same ailment(s) in different countries. In other instances, different species are used in treating the same ailment(s). This could imply that they possibly contain the same bioactive secondary metabolites.

The roots are the parts that are mostly used in preparing traditional remedies. However, the leaves and the stem-bark are also used, though less frequently.

Table 1.1 Traditional medicinal uses of *Maytenus* species.

***Maytenus* species**	**Medicinal uses**	**Country**	**References**
M. acuminata (L.f.) Loes	Stomach ailments	South Africa	POOLEY (1993)
M. arbutifolia (A.Rich.) R. Wilczek	Malaria Fevers	Kenya	GAKUNJU *et al.* (1995)
M. aquifolium Mart.	Ulcers	Brazil	GONZÁLEZ et *al.* (2001) CORDEIRO *et al.* (1999)
M. buchananii (Loes) R. Wilczek	Stomach upsets Leukaemia	Eastern-Africa	KOKWARO (1976)
M. heterophylla (Eckl and Zeyhl.) N.K. Robson	Diarrhoea	Southern-Africa	WATT and BREYER-BRANDWIJK (1962)
	Coughs Colds Haemorrhoids Measles Venereal diseases	Botswana	HEDBERG and STAUGARD (1989)
	Antihelmintic Hernia Syphilis	Eastern-Africa	KOKWARO (1976).
	Tonic	Kenya	BEENTJE (1994)
	Dysmenorrhoea	Zambia	HEDBERG and STAUGARD (1989)

Table 1.1 Contd.

***Maytenus* species**	**Medicinal uses**	**Country**	**References**
M. ilicifolia Mart.	Ulcers	Brazil Peru	GONZÁLEZ *et al.* (2001) CORDEIRO *et al.* (1999)
M. putterlickiodes (Loes) Exell and Mendonca	Hookworm Aphrodisiac	Eastern-Africa	KOKWARO (1976)
M. senegalensis (Lam.) Exell	Chest pains Rheumatism	Kenya	GACHATHI (1989)
	Snake bite Eye infections Rheumatism Aphrodisiac	Eastern-Africa	KOKWARO (1976)
	Pneumonia Tuberculosis Venereal diseases Epilepsy Pain Constipation Infertility Sore throat Earache	Southern-Africa	WATT and BREYER-BRANDWIJK (1962) ROODT (1998) VAN WYK *et al.* (2000)

Table 1.1 contd.

Maytenus species	Medicinal uses	Country	References
M. *senegalensis* (Lam.) Exell	Tumours Dysentery Snakebites	Sudan	HUSSEIN *et al.* (1999)
	Malaria	Tanzania	GESSLER *et al.* (1994)
M. undata	Tonic Syphilis Urethra infections	Eastern-Africa	KOKWARO (1976)

1.2.2 Pharmacological effects

Bioactivity in *Maytenus* species has been attributed to various groups of secondary metabolites such as triterpenes and several types of alkaloids (HUTCHINGS *et al.* 1996).

Compounds exhibiting anticancer properties have been identified in some *Maytenus* species. Maytensine, an ANSA macrolide type of alkaloid (ADDAE-MENSAH 1992) with anti-leukaemic properties has been isolated from *M. ovatus* and *M. serrata* (HARBONE and BAXTER 1993), *M. emarginata* (KUO *et al.* 1990) and *M. buchananii* (SEKAR *et al.* 1996). HARBONE and BAXTER (1993) have described maytansine as the most potent plant anticancer agent. However, its harmful side effects such as gastro-intestinal, hepatic and neurological disorders have currently deterred its use in humans but its anticipated that synthetic or semi-synthetic derivatives may offer some hope (EVANS 1996).

Other alkaloids exhibiting anti-cancer properties include macrocarpin, from *M. macrocarpa* (CHAVEZ *et al.* 2000) and emarginatine A and B from *M. emarginata* (KUO *et al.* 1990). Triterpenes such as maytenfolic acid from *M. diversifolia* have also been found to possess anticancer properties (NOZAKI *et al.* 1990).

Various workers have isolated compounds showing antibacterial activity from several *Maytenus species.* Dihydrogarofuran alkaloids and maytenfolic acid from *M. heterophylla* and *M. arbutifolia* (ORABI *et al.* 2001), Pheno-nor-triterpenes from *M. canariensis* (GONZÁLEZ *et al.* 1996; HERRERA *et al.* 1996) and *M. undata* (MUHAMMAD *et al.* 2000) had antibacterial activity.

In vitro antiplasmodial activity of *M. senegalensis* root bark and stem bark (GESSLER *et al.* 1994; EL TAHIR *et al.* 1999) and *M. arbutifolia* roots (GAKUNJU *et al.* 1995) have been exhibited. *In vivo* antiplasmodial activity was found in extracts of *M. senegalensis* (GESSLER *et al.* 1995). Pristemerin, a pentacyclic triterpene of the friedelane type was responsible for antiplasmodial activity in *M. senegalensis* (EL TAHIR *et al.* 2001).

South American *Maytenus* species are traditionally used to treat gastrointestinal ailments such as ulcers and as analgesics (Table 1.1). *In vitro* and *in vivo* anti-ulcer and analgesic properties of *M. aquifolium* (GONZÁLEZ *et al.* 2001) and *M. Ilicifolia* (CORDEIRO *et al.* 1999) have been shown. Flavonoid glycosides (LEITE *et al.* 2001) and triterpenes such as friedelan-3-ol and fridelin (PEREIRA *et al.* 1993; CORDEIRO *et al.* 1999) are responsible for anti-ulcerogenic effects in *M. aquifolium* and *M. Ilicifolia*. Therapeutic action of triterpenes in treatment of ulcers is through the enhancement of synthesis of mucus and maintenance of prostaglandin levels (GONZÁLEZ *et al.* 2001).

In vitro antileishamanial activity (EL TAHIR *et al.* 1998) and moderate affects against HIV-1 protease (HUSSEIN et *al.* 1999) have been exhibited by stem-bark extracts of *M. senegalensis.*

1.2.3 Propagation and conservation

The conservation status of many medicinal plants is unknown (KOKWARO 1993) and that of traditionally used *Maytenus* species is no exception. However, it is reported that despite the wide distribution range of *M. senegalensis* in East Africa (ROBSON *et al.* 1994; MARSHALL 1998) and Kenya in particular (BEENTJE 1994), its population is on the decline in Kenya (AKETCH 1992), Uganda (MARSHALL 1998) and Tanzania (TESHA 1991).

Overutilization of *M. senegalensis* due to its popularity in traditional medicine, habitat destruction (AKETCH 1992) and selective destruction as a source of plant parts for pharmacological screening in international laboratories (TESHA 1991) could have led to the depletion. Kenya is listed as the origin of *M. senegalensis* stem bark and root bark for screening in overseas laboratories (*http://www.worldbotanical.com/WBAMED2. HTM*). There are no reports of any propagation efforts on *M. senegalensis* (ROODT 1998). However, *M. canariensis* ((MENDEROS-MOLINA 2002), *M. emarginata* (RATHORE *et al.* 1992) and *M. ilicifolia* (PEREIRA *et al.* 1995) have all been successfully micropropagated.

1.3 BOTANICAL DESCRIPTION OF *M. SENEGALENSIS*

Maytenus senegalensis whose synonymns include: *Celastrus senegalensis* Lam., *Gymnosporia senegalensis* (Lam.) Loes var *angustifolia* Engl. and Loes and *Gymonosporia coriacea* (Guillll. and Perr.) Eggeling (ROBSON *et al.*, 1994) is a shrub or a small tree (Plate 1.1) that grows to a height of 1-9 meters. It has a fissured, greyish brown bark, often with straight rigid 5 cm long spines, which are either axillary borne, or may be found on short side branches. The young branches are markedly reddish.

The leaves are alternately borne or in fascicles, bluish–green in colour, oblong to obovate in shape and have a tapering base and a rounded, often notched apex. They measure 2-12 cm long by 0.4-6.5 cm wide, are glabrous and have a sub entire or a

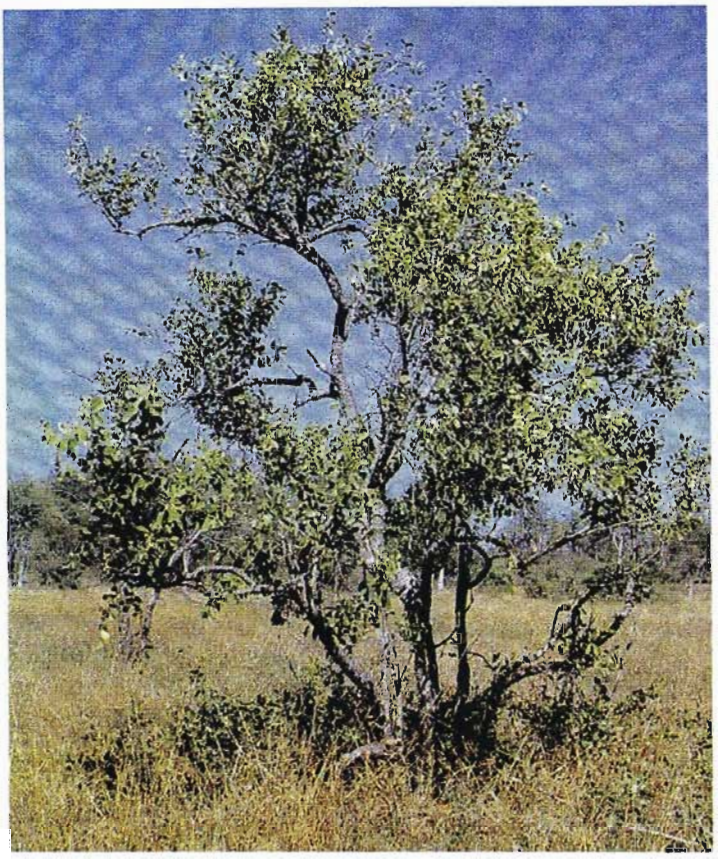


Plate 1.1 *Maytenus senegalensis* (Lam.) Exell (Synonymn: *Gymnosporia senegalensis* (Wight and Arn.) Hook, f.). (From VAN WYK *et al.* 2000).

serrulate (shallow rather rounded teeth) margin. The flowers are greenish, white or cream, in dense axillary clusters, 1-4 cm long with petals measuring 1-3.5 mm long. The separate flower sexes appear on different trees and rarely on the same tree. The fruit is a two-lobed capsule, greenish with a red flush when mature and occasionally become reddish-brown. The seeds measure 2-6 mm long and have a rose pink or yellow aril (BEENTJE 1994).

According to ROBSON *et al.* (1994), *M. senegalensis* is sometimes confused with *Maytenus heterophylla*. However, it can be identified easily on the basis of the presence of unlined reddish or glaucous (hairless) young shoots.

Some of the local names for *Maytenus senegalensis* in Kenya include:
Mdunga-ndewe, Mnyonyao (Kiswahili), Muenyuke (Kikuyu), Kivunda-ngiti (Kamba), Ol-lalmoronaili (Maa), Mathari, Nyandema (Luo). *M.senegalensis* occurs in wooded or bushed grasslands and in semi-arid riverline areas and less often in rocky bushland from sea level to 2,100 metres (BEENTJE 1994; DHARANI 2002).

1.4 AIMS OF THE STUDY

It is evident that *M. senegalensis* is a popular medicinal plant in eastern and southern Africa countries (Table 1.1) and it has attracted pharmacological attention at the international level as well (TESHA 1991). There is need therefore to validate some of its traditional medical uses, develop rapid *in vitro* propagation techniques as a conservation option and explore the potentials of alternative sources of bioactive secondary metabolites such as *in vitro* root cultures.

The aims of this study were therefore to:

1. Screen various parts of *M. senegalensis* alongside other Kenyan medicinal plants for *in vitro* antibacterial and anti-inflammatory activities;
2. Isolate the antibacterial compound(s) from *M. senegalensis* root-bark using bioactivity guided fractionation;
3. Develop a viable micropropagation protocol for *M. senegalensis* using in *vitro* germinated seedlings as the source of explants;
4. Optimise the factors (environmental and physiological) influencing root initiation and growth of *M. senegalensis in vitro* shoots; and
5. Establish an in *vitro* root culture of *M. senegalensis* as potential alternative sources of antibacterial compounds.

CHAPTER 2

LITERATURE REVIEW

2.1 TRADITIONAL MEDICINE: A GLOBAL PERSPECTIVE

Traditional medicine is a vague term, loosely used to distinguish culture-bound health practices from modern or allopathic medicine (BANNERMAN 1983). However, traditional medicine can simply be defined as the application of indigenous beliefs, knowledge, skills and cultural practices concerned with human health (AREGBEYEN 1996). Some frequently used synonyms are indigenous, unorthodox, alternative, folk, ethno, fringe and unofficial medicine and healing (BANNERMAN 1983; BUCKMAN and SABBAGH 1993; PANTANOWITZ 1994). The use of the term "modern" in reference to non-traditional medicine was advanced on the basis of its tendency to quickly adopt scientific and technological innovations (PANTANOWITZ 1994), an advantage that is employed by traditional medicine to a very small extent (SINDIGA 1995).

Both traditional and modern medicines have a common objective of healing and preventing disease. However, the two systems differ in the concept of the cause of disease, their approach to healing as well the methods of treatment (SOFOWORA 1982). Although traditional medicine mostly employs the use of plants or plant parts (ABEBE and AYEHU 1993), animals, animal parts or minerals (KOUMARÉ 1983; NETO 1999) may be used

Traditional medicine is widespread throughout the world and in fact, it was the only form of health care before the incorporation of modern science into medicine in the early part of the 20th century (BANNERMAN 1983). Its part of the traditions of each country and its practices are handed down from generation to generation.

Despite the efforts by governments all over the world to provide modern health care to their citizens, the use of traditional medicine has persisted. This scenario is especially evident in the rural parts of the developing world where it is estimated that up to 80% of the population rely on traditional medicine for their health care needs (BANNERMAN 1983). This phenomenon can be attributed to economic and cultural factors (AKETCH 1992

In the industrialized countries, traditional medicine practices slumped for most part of the 20th century. However, there has been a resurgence of interest in the recent past (ZHANG 1996; CRAKER 1999) despite the widespread availability of modern medicine (BANNERMAN 1983). Various forms of traditional medicine such as herbal medicine, aromatherapy and acupuncture are widely practised (BANNERMANN 1983).

A survey carried out in 1992 showed that a third of the population in the United states of America made use of at least an alternative form of treatment. In Europe, 60% of the Dutch and the Belgians were willing to pay extra health insurance for alternative medicine while 74% of the British favoured complimentary medicine being available in the health services (ZHANG 1996). Increased reliance on alternative forms of health care can be attributed to factors such as awareness of the shortcomings of conventional drugs (EVANS 1996) and high health care costs (ROBBINS 1997) among other factors.

In many Asian countries, traditional medicine has existed alongside modern medicine over the years. In Japan for instance, 60-70% of modern doctors prescribe some form of traditional drugs. In China, traditional medicine is extensively used (LEWIS and ELWIN-LEWIS 1977). It is estimated that it accounts for 40% of all health care delivered and it is used to treat about 200 million people annually (WHO 2002). Other Asian countries where traditional medicine has been successfully integrated with modern medicine include India, South Korea and Vietnam (WHO 2002).

Traditional medicine is also widely used in the countries of South America. Seventy-one and 40% of the population in Chile and Columbia respectively use traditional medicine (WHO 2002).

2.2 AFRICAN TRADITIONAL MEDICINE

It is widely acknowledged that about 80% of the African population relies on traditional medicine for their health care needs (KOUMARÉ 1983). African traditional medicine can be described "as the sum total of all knowledge and practices whether explicable or not, used in diagnosing, preventing or eliminating physical, mental or social imbalance and which rely exclusively on practical experience and observations handed down from generation to generation, whether verbally or in writing" (SOFOWORA 1982).

The concept of disease causation among the Africans reflects their culture (KOKWARO 1995; SINDIGA 1995) and can be grouped into two categories. The first category are the "naturally" caused or God-given diseases whose cause(s) can be attributed to tangible materials and the patient can easily describe them to the traditional healer. They are hence regarded to as normal or minor. The traditional healer can treat such diseases in strictly physical terms using material substance such as plant, animal parts and/or minerals.

The second category includes diseases, which are severe and persistent. Their cause(s) are attributed to "unnatural" or "intangible forces". The advent of such diseases implies that a hostile person could be using supernatural powers against the patient or that the victim may have breached the social-religious obligations and hence incurred the wrath of the ancestors (the living dead) or the deity. Treatment of such diseases goes beyond the use of tangible materials and borrows resources from the immaterial world, which could include a wide range of charms, amulets, spells and incantation (GOOD and KIMANI 1980).

The majority of the people in African countries lack accessibility to modern medicine for economic reasons and even when available, it is of low quality at unacceptable levels (ENWEREJI 1999). The number of Traditional Medical Practitioners (TMPs) to the population is much greater than the number of doctors. For instance, KOUMARÉ (1983), reports that there is one Traditional Medical Practitioner to every five hundred inhabitants compared to one doctor to every 40, 000 inhabitants. Besides, African traditional medicine employs a holistic approach whereby the psychosocial and physical aspects of disease are addressed during treatment. It is thus popular and it is sometimes sought even when modern medicine is accessible (SINDIGA 1994).

The colonial administrators suppressed traditional medicine for most part of the last century forcing the practice to go underground (ATTISO 1983) in most countries. This was as a result of its association with witchcraft (YONDER 1982). Its belief in supernatural forces as causative agents of disease did not augur well with the early Christian missionaries who thus discouraged the practice (SINDIGA 1995; MARSHALL 1998). However, this scenario has changed and many African governments have recognised traditional medicine as an official practice. However, traditional medicine and modern medicine do not cooperate but rather exist side-by-side (SINDIGA 1994).

The World Health Organisation (WHO) in its efforts to provide health care for all passed a resolution at Alma-Atta in 1976 in which it urged member countries especially those in the developing world to incorporate the good aspects of traditional medicine into their primary health care policies (AKERELE 1991). As a result, many countries are currently in the process of integrating traditional medicine with modern medicine in their national health policies (SOFOWORA 1982; MARSHALL 1998).

Traditional Medical Practitioners (TMPs) who are popularly referred to as traditional healers are people whom the community in which they live recognizes as competent enough to provide health care (KOUMARÉ 1983; KOKWARO 1995). Their knowledge and skills are based on years of empirical observation (SOFOWORA 1982; SINDIGA 1995) and are handed down from generation to generation, through word of mouth

(KOKWARO 1995). Traditional Medical Practitioners can be classified into various groups according to their expertise and usually one can fit into one or more categories. There are the herbalists, diviners, spiritualists, traditional surgeons, traditional birth attendants and bonesetters (SOFOWORA 1982).

2.3 TRADITIONAL MEDICINE IN KENYA

Despite the introduction of modern medicine to Kenya in the last two decades of the 19th century (NYAMWAYA 1986), the use of traditional medicine has continued (AKETCH 1992). Precise statistics of the population relying on traditional medicine are lacking. However, estimates indicate that 70-90% of the rural population rely on traditional medicine for their health care (ODERA 1997). These estimates could go to 100% among the pastoral communities who reside in the arid and semiarid areas where modern medicine is restricted to immunization and malaria control programmes only (FRATKIN 1996). However, in most cases, medical pluralism exists whereby both modern and traditional medicine exists side by side especially in the urban areas (GOOD and KIMANI 1980; SINDIGA 1994).

The last three decades have witnessed a very great upsurge of interest in traditional medicine as is evidenced by the increased activities of Traditional Medical Practitioners and an increase in trade on medicinal plants (AKETCH 1992). Thus, medicinal plants, which are mostly used in preparing traditional remedies, are in high demand in the health care systems when compared with modern medicines (AKETCH 1992). Estimates indicate that over 400 plant species are used in the management of common diseases in Kenya (KOKWARO 1976).

The government of Kenya has acknowledged the major role played by traditional medicine. Consequently, preparation of a bill to be tabled in parliament is underway. It is anticipated that existence of the bill will pave way for official integration of traditional medicine with modern medicine in the national health care system (OKWEMBA 2002).

2.4 PLANTS AS THERAPEUTIC AGENTS

2.4.1 Plants in traditional medicine

There is enormous evidence from ethnomedical literature that most of the traditional remedies are prepared from plants in general (BLOCK 1986; COTTON 1996; ERDELEN *et al.* 1999) and higher plants (angiosperms) in particular (ABEBE 1987). Medicinal plants thus form the backbone of traditional medicine (ATTISO 1983; FARNSWORTH 1994). Reports of plants use in therapy date back to very far (SOFOWORA 1982; PANTANOWITZ 1994; BATANOUNY 1999). For instance, there are reports to the effect that the Egyptians were using sycamore bark and oil from cedar for medicinal purposes more than five thousand years ago (HOLDSWORTH 1986).

Traditional remedies prepared from plants are used in treatment of many ailments for instance, bacterial infections, diarrhoea, skin infections, parasitic infestations, as poultices, in dental hygiene, as birth aids, contraceptives among other ailments. Medicinal plants also find application as sources of psychoactive agents among Traditional Medical Practitioners (TMPs) who specialise on treatment of diseases whose causes are perceived to be supernatural (COTTON 1996).

Plant species from a wide range of plant families are used in traditional medicine. However, the exact number of plant species used in traditional medicine has not been precisely determined, an anomaly that could be due to the incompleteness of the lists provided by different countries (HEYWOOD 1999). However, FARNSWORTH and SOEJARTO (1991) estimated the number to be about 28% of the total world flora. Considering that the total world flora is estimated to be about 250,000 plant species, the number of medicinal plant species could thus be between 70,000-80,000.

India and China are reported to be the worlds' major users of medicinal plants. For instance, 7,000 plant species are used in Indian traditional medicine while over 5,000 are used in Chinese traditional medicine (ZHANG 1996). Medicinal plants are also

widely used in African traditional medicine (WATT and BREYER-BRANDWIJK 1962; KOKWARO 1976; GELFAND *et al.* 1985; ABEBE 1987; NYAZEMA 1987; MARSHALL 1998; ENWEREJI 1999).

In the industrialised countries, there has been an increase in reliance of semi-processed or unprocessed plant extracts, which are used as home remedies or sold as over-the-counter medication. Most of these medicinal plants are obtained from foreign countries with China accounting for a trade on medicinal plants worth US dollars 1 billion. In the United States of America, imports of medicinal plants have increased by a value of 40% in the last decade (CRAKER 1999). Germany is the worlds leading importer of medicinal plants (LANGE 1997).

2.4.2 Plants in modern medicine

The advent of organic chemistry in the last two decades of the nineteenth century facilitated the application of medicinal plant extracts into modern medicine. As a result, pure compounds that were safe and effective could be isolated from plants and developed into drugs. A survey carried out in the United States of America between 1959 and 1976 showed that 25% of the drugs dispensed in community pharmacies were derived from higher plants (FARNSWORTH and SOEJARTO 1985). It is also acknowledged that 90 medicinal plant species were involved in the production of the 119 chemical extracts that are useful as modern drugs and are still extracted from plants (FARNSWORTH 1994).

Medicinal plant extracts can be utilized in modern medicine in various ways. They can for instance directly be used as therapeutic agents (FARNSWORTH and SOEJARTO 1991). This is especially so in cases where chemical synthesis proves to be very difficulty or is not cost effective. Examples of such drugs include vincristine: an alkaloidal drug obtained from *Catharanthus roseus* for treatment of childhood leukaemia, morphine: obtained from *Papaver somniferum* for treatment of deep pain as in cancer patients, reserpine: from *Rauvolfia* spp for treatment of mild hypertension and as a

tranquilliser and D-tubocurarine, an alkaloid obtained from *Chondrodendron tomentosum* which has muscle-relaxant properties, hence is useful in surgery.
In other instances, plant constituents may be used as starting points for the synthesis of useful drugs (PRINCIPE 1991). A good example of this is the synthesis of steroidal drugs (corticosteroids, sex hormones, oral contraceptives and anabolic agents) from sapogenins obtained from plant species. Diosgenin obtained from tubers of some species of *Dioscorea* was widely used in the past as a starting material for the production of various steroidal drugs (HUSAIN 1991; BAQUAR 1992).

Technological advancement in synthetic chemistry has brought another dimension in the use of plants in modern medicine. Bioactive compounds from plants serve as models for the synthesis of new therapeutic compounds. This is especially fashioned in a bid to enhance activity or when the natural compounds have adverse side effects (FARNSWORTH 1994). Examples of such drugs include: salicylic acid obtained from *Salix* species from which aspirin was synthesized or chloroquine, which was synthesized from quinine obtained from *Cinchona* species. Synthesis of new compounds is also a better option when availability or collection of plant material is not reliable (PRINCIPE 1991).

Medicinal plants also find use in modern medicine by serving as research tools but not directly as therapeutic agents (FARNSWORTH and SOEJARTO 1991). This is especially true in instances where the bioactive compound has adverse side effects and it cannot be artificially synthesized. Incorporation of such compounds as a prescription drug is thus not possible. Examples of such drugs include the more than 50 compounds that have been ascertained to have anti-cancer properties but because of their adverse side effects, they are only used in research to facilitate the understanding of biochemical processes and their mechanisms of action (PRINCIPE 1991).

2.4.3 Negative aspects of traditional medicine

Like any other system, traditional medicine has its drawbacks, which to a given extent limit its impact (SOFOWORA 1982). Some of its drawbacks include:

• Toxicity of some medicinal plants: Some of the medicinal plants that are widely used in traditional medicine are known to contain toxic substances (NYAZEMA 1987). Examples of such compounds are the carcinogenic pyrolizidine type of alkaloids, which are found in species of the genera *Strychnos* (Loganiaceae), *Crotalaria* and *Senecio* (Asteraceae) (KOFI-TSEKPO 1997). High prevalence of liver, kidney and lung diseases in some localities of Ethiopia is for instance attributed to the widespread use of traditional remedies prepared from members of *Crotalaria*, *Senecio* and *Cynoglossum* (SHROENTAL 1972).

• Lack of scientific proof of its efficacy: This is probably the severest criticism against traditional medicine (SOFOWORA 1982). Claims of efficacy of traditional remedies are limited to the Traditional medical practitioners and their clientele. This is because the remedies have not been subjected to controlled scientific studies.

• Imprecise nature of diagnosis: Traditional Medical Practitioners lack scientific equipments and the skills to facilitate proper diagnosis of disease (HOSTETTMANN and MARSTON 1986). This is especially problematic in ailments that have similar symptoms for instance, what the Traditional Medical Practitioner may diagnose as stomach trouble may be due to indigestion, stomach ulcers or cancer of the stomach, ailments that have very different aetiologies and hence would need different types of remedies (SOFOWORA 1982).

Most of these drawbacks can be overcome with proper integration of traditional medicine with modern medicine. However, toxicological studies should be intensified since some of the toxic effects are long term.

2.4.4 Ethnobotanical approach in drug discovery

Selection of candidate plant species for biological activity screening can be approached in various ways. They include: a chemotaxonomic approach which is based on correlation between plant taxonomy and the occurrence of specific chemical compounds (RASOANAIVO and RATSIMAMANGA-URVERG 1993), random selection approach in which plants or plant parts are randomly selected and then subjected to biological screening (FARNSWORTH 1994) and ethnobotanical approach in which plants used in traditional medicine are screened for biological activity (HEDBERG 1987)

Several workers have reported that the ethnobotanical approach is the best option as it is faster and cheaper (HOSTETTMANN and MARSTON 1986; PLOTKIN 1991). The chances of obtaining worthwhile results are also higher when this approach is used (PURDUE *et al.* 1970; SOFOWORA 1982; HOSTETTMANN and MARSTON 1986). This recommendation is given more weight by the fact that many modern drugs were discovered on the basis of their traditional folkloric uses (HEDBERG 1987; FARNSWORTH and SOEJARTO 1991; COX 1994; PRANCE 1994). It should hence be incorporated as a tool in validating the use of medicinal plants in traditional medicine especially in developing countries where traditional medicine is in the process of being integrated with modern medicine in the national healthcare policies (JÄGER *et al.* 1996).

2.5 CONSERVATION OF MEDICINAL PLANTS

There are several reasons as to why medicinal plants should be conserved .The major role played by medicinal plants in traditional and modern medicine has been demonstrated and hence they should be conserved for use by future generations (ABEBE 1987). Besides, only a small fraction of potential medicinal plants have been subjected to intense investigation for bioactive secondary metabolites, hence potential cures could still be unexplored (PANTANOWITZ 1994)

However, high population growth rates especially in the developing countries have led to increased demand for medicinal plants and destruction of habitats in which they occur (HAMANN 1991; AKETCH 1992; PUSHPANGANDAN and THOMAS 1999). In the past, traditional medicine practise was the domain of Traditional Medical Practitioners and conservation practises were in place to ensure that the medicinal plants were conserved and used in a sustainable manner (ODERA 1997). However, in order to meet the rising demand and as a result of unemployment, a form of trade on medicinal plants has evolved at local, regional and international level (AKERELE 1991). The major drawback about this trade is that the people involved in gathering and collecting the medicinal plant parts are ignorant of the "traditional" conservation measures and hence many medicinal plants have been killed as a result of practises such as excessive debarking and root removal (CUNNINGHAM 1991).

Some medicinal plants have multiple uses, which further leads to depletion of their populations. For instance, *Prunus africana*, which is the source of extracts that are used in treatment of prostate problems, is also widely sought as a source of timber (LEAKEY 1997). Another example is *Olea europaea* ssp *africana*, which has a wide application in traditional medicine but its also very popular as a source of firewood and charcoal in Kenya (MAUNDU *et al.* 2002).

Conservation of medicinal plants can be achieved in various ways. Utilization of plant parts whose removal does not threaten the survival of the plants and exhibits bioactivity could be encouraged. For instance, substitution of leaves for roots or stem-barks (ZSCHOCKE *et al.* 2000).

Incorporation of indigenous medicinal plants in afforestation and re-afforestation programmes would ensure that the gene pools are sustained (AKETCH 1992). Cultivation of medicinal plants in small-scale farms would ease pressure off wild stocks (BONATI 1991; CRAKER 1999) as well as provide a source of livelihood for the unemployed people who are involved in destructive harvesting techniques. The major drawback in this regard is that propagation techniques for most medicinal plants are still

unknown and thus there is need to develop rapid means of propagation (KOKWARO 1991).

2.6 TISSUE CULTURE

Plant tissue culture techniques involve aseptic cultivation of cells, tissues and organs isolated from the mother plant in an artificial medium (SEABROOK 1980; THORPE 1990; GEORGE 1993) under controlled light and temperature. Generally the medium consists of five classes of substances viz-inorganic macro- and micronutrients, carbon/energy source, vitamins and plant growth regulators (GAMBORG 1975; THORPE 1990).

The fundamental basis of plant cell tissue culture is based on the fact that an individual cell has the capacity to regenerate into a whole organism of which it was part (totipotency). This notion was postulated way back in the mid 19th century (CONSTANTIN 1978). The first trials to demonstrate totipotency were carried by Haberlandt at the beginning of the 20th century and although he was able to keep the cells alive, he was unable to induce cell division (COLLINS and EDWARDS 1998). However, White in the 1930's had a breakthrough when he cultured tomato roots indefinitely in a simple medium containing inorganic salts, sucrose and yeast extracts (COLLINS and EDWARDS 1998). Culture of cambium and parenchyma of stems, roots and tubers was also achieved at around the same time by Gautheret (CONSTANTIN 1978).

Rapid progress in tissue culture techniques was made upon the discovery of plant growth regulators in the 1950's (PIERIK 1987). The development of defined media and special equipments has further facilitated refinement of the techniques (CONSTANTIN 1978).

Plant cell and tissue culture techniques find application in rapid mass propagation of useful plant genetic resources for commercial exploitation, for conservation purposes,

enhance production of disease free plant material for increased yields and facilitate exchange between countries, facilitate breeding/genetic improvement, preservation of germplasm (cryopreservation) and can be used as alternative sources of bioactive secondary metabolites (DE FOSSARD 1977; JAWORSKI 1978; SEABROOK 1980; WILKINS and DODDS 1983; ELHAG 1991; GEORGE 1993,1996; KOKWARO 1993; ABO EL-NIL 1997)

2.6.1 Micropropagation

Micropropagation can be defined as the process of multiplying plants by non-sexual methods under *in vitro* conditions (MURASHIGE 1974, 1977) through direct or indirect organogenesis (DODDS 1983; GEORGE 1993). Direct organogenesis is achieved through induced morphogenesis of pre-existing buds such as in the shoot tip, node or apical meristem or non-meristematic tissues to give rise to axillary or adventitious shoots respectively. Indirect organogenesis is achieved through a callus phase, which can then be induced to form adventitious shoots.

Although it is possible to propagate many plant species using conventional techniques using seeds and vegetative cuttings, there are limitations, which make micropropagation a better option. Propagation using seeds gives rise to genetically heterozygous individuals especially in woody plant species resulting in enormous variation in the standard of the material produced (WILKINS and DODDS 1983; WAINWRIGHT and ENGLAND 1987). Production of few viable or recalcitrant seeds also hampers the use of seeds as propagules (ABO EL-NIL 1997). Conventional methods using vegetative cuttings on the other hand are slow, difficult and expensive and only a small number of plants can be produced at a given time (BENGOCHEA 1983). The main intent of micropropagation is to ensure rapid mass production of plants with uniform desired qualities (MURASHIGE 1977).

There are various ways through which new plants can be obtained by using tissue culture (MURASHIGE 1977). They include:

1. Somatic cell embryogenesis whereby a plant tissue is induced to form somatic embryos that develop into adventitious shoots. *In vitro* plant propagation is fastest using this technique and a very large number of plants can be produced within a very short time (DODDS and ROBERTS 1985). However, this method is restricted in applicability (MURASHIGE and HUANG 1987) in that some genera fail to respond like in oak (*Quercus* spp) (WILHELM 2000). However, successful production of plants was achieved in *Podocarpus henkelii* (KOWALSKI and VAN STADEN 1998). There are possibilities of production of aberrant plants when production is through adventitious methods (MURASHIGE and HUANG 1987).

2. Production of adventitious shoots, which arise in tissues other than the normal leaf axil region such as the stem, bulbs, corms etc. Production of adventitious shoots can be direct or through an intermediary callus phase. Plants produced through a callus phase are vulnerable to genetic instability and regeneration capacity of the shoots declines with repeated subcultures (MURASHIGE 1977).

3. Production of axillary shoots, which emerge from the leaf axil. Plant production is slowest using this method. However, it enjoys the widest applicability in most genera and the plants produced are less variable genetically (GEORGE 1993). This method has been successfully used for mass production in *Uvaria picta* (ANAND *et al.* 1998). Nevertheless, repeated subculture of *Vitis vinifera* led to changes in morphology of leaves (MULLINS *et al.* 1979).

Five stages through which the process of plant multiplication *in vitro* proceeds have been documented as follows:

Stage 0: Treatment and preparation of stock plants (DEBERGH and MAENE, 1981)
Stage I: Establishment of an aseptic culture
Stage II: Multiplication of propagules
Stage III: Preparation for re-establishment in the soil (MURASHIGE 1974, 1977).

Stage IV: Transfer to the external environment (GEORGE 1993).

One of the major limitations to developing an efficient micropropagation protocol is microbial contamination of explants (GARTON *et al.* 1983; PIERIK 1987). Explants should thus be sourced from stock plants in which contamination is minimal. Various factors such as the genotype, age of the stock plant, the position/age and type of the explant are known to affect the ability to regenerate (GEORGE 1993). Stock plants can be placed in the greenhouse if they are herbaceous or woody seedlings where they are treated with bactericides and fungicides such as 0.1% Benomyl (PIERIK 1987). In cases where decontamination is a major problem, *in vitro* germinated seedlings can be used as the source of explants (JOHNSON *et al.* 1997). The only limitation when *in vitro* germinated seedlings are used is that their field performance is still unknown at that stage.

The establishment of an aseptic culture is crucial and various agents such as 70% alcohol, sodium hypochlorite (NaOCl), calcium hypochlorite ($CaOCl_2$) and Mercuric chloride ($HgCl_2$) are widely used to decontaminate the explants (DODDS and ROBERTS 1985). However, the efficacy of a sterilant, optimal concentration and the duration of exposure are all genotype and explant dependant (MURASHIGE 1977). In *Rhanterium epapposum,* (ABO EL-NIL 1997) exposure of explants in 1% NaOCl for 10 min was effective while 0.8% of NaOCl for 15 min was required for *Litsea cubeba* (MAO *et al.* 2000). Mercuric chloride is toxic to man and hence it should be used with caution at concentrations of 0.01-0.5% for 10-15 minutes (PIERIK 1987).

In order to reach all the explant surfaces, the explants may be dipped in 70% alcohol for a minute or two before they are decontaminated. Inclusion of a wetting agent such as Tween-20 or Tween-80 lowers the surface tension facilitating better surface contact (PIERIK 1987). The explants should then be thoroughly rinsed in not less than three changes of sterile distilled water, each lasting 5 min.

The explants that should be free from obvious microbial contamination are then aseptically placed on a medium and some form of growth should follow (GEORGE 1993). Growth can be in the form of callus or proliferation of adventitious or axillary shoots.

Once an aseptic culture has been established, the propagules are then multiplied. The explants can either be primary or secondary (obtained from *in vitro* shoot clusters). The ability of explants to regenerate is genera or genotype dependent. Shoot tips were the most regenerative explants in *Bacopa monnieri, Paederai foetida* and *Centella asiatica* (SINGH *et al.* 1999) while nodal explants were the most regenerative explants in *Litsea cubeba* (MAO *et al.* 2000).

2.6.1.1 Media and culture conditions

Shoot initiation, proliferation and *in vitro* rooting (induction and growth) to a large extent is dependent on the media and the cultural conditions the explant is subjected to (GEORGE 1993). The cultural environment influences the physiology and anatomy of cultures (LEE *et al.* 1985). Research efforts over a long period of time have led to the development of several types of media. However, the suitability of each medium is species-variable (GEORGE 1993). The most widely used medium is that of Murashige and Skoog (MS) (MURASHIGE AND SKOOG 1962). Woody plant media (WPM) (LLOYD and McCOWN 1981) is also widely used, especially in woody plant species (GEORGE 1993).

In *Litsea cubeba* for instance, WPM-treated shoots were significantly longer compared to MS-treated shoots although there were no significant differences in the number of shoots produced per explant (MAO *et al.* 2000). However, WPM promoted shoot necrosis in *Cercis canadensis* var *mexicana* (MACKAY *et al.* 1995). The strength of the media may also influence organogenesis (PIERIK 1987). This is especially evident while attempting to induce *in vitro* rooting (SOMMER and CALDAS 1981; GEORGE 1993).

Half strength MS salts promoted rooting better than full strength MS salts in grape (LI and EATON 1984).

It's a well-known fact that plant growth regulators (PGRs) play a major role in plant growth and development (EVANS 1996). Cytokinins and auxins are the major groups of plant growth regulators involved in *in vitro* plant propagation (DODDS and ROBERTS 1985). Generally, cytokinins promote cell division while auxins promote cell elongation. However, a balance between cytokinins and auxins is needed in the regulation of cell division, cell elongation, cell differentiation and organ formation (DODDS and ROBERTS 1985). For instance a higher level of cytokinins to auxins favour shoot production and vice versa for root production while nearly equal levels favour callus formation (GEORGE 1993).

The effects of PGRs and the concentration used for shoot initiation, proliferation and *in vitro* rooting (induction and growth) is species dependent (GILES and SONGSTAND 1990; COLLINS and EDWARDS 1998). In *Albizia chinensis*, 6-benzylaminopurine (BA) was superior in shoot production compared to kinetin and thidiazuron (TDZ) (SINHA *et al.* 2000). Combination of BA and Indole-3-acetic acid (IAA) promoted shoot production in *Eryngium foetidium* than BA alone (AROCKIASAMY and IGNACIMUTHU 1998). Although higher levels of cytokinins are widely used during shoot induction and multipliction (GEORGE 1993), there are exceptions. In *Aloe barbadensis* for instance, α-Napthaleneacetic-acid (NAA)-treated explants produced the highest number of shoots (MEYER and VAN STADEN 1991). Auxins such as IBA, IAA and NAA are widely used for production of roots *in vitro* (GEORGE 1993).

A carbon/energy source should be incorporated in the medium (PIERIK 1987). Sucrose at 3% is mostly used (MURASHIGE 1974) but other carbon sources such as glucose at 2-4% are used (GAMBORG 1975). Carbohydrates such as sucrose and glucose also play the role of an osmoticum in the medium (GEORGE 1996; KUSIKARI *et al.* 2000).

The pH of the medium should be such that it does not disrupt the functioning of plant cell membranes or the buffered pH of the cytoplasm (GEORGE 1993; LEE *et al.* 1988). A pH value of 5.5-5.8 is widely used (MURASHIGE 1977). High concentration of hydrogen ions may affect the stability of IAA, precipitation of phosphates and heavy metals or gelatinisation of agar and destruction of thiamin during autoclaving (BUTENKO 1968; GEORGE 1993).

The physical nature of the medium influences the rate at which cultures grow (LEE *et al.* 1988; GEORGE 1993). Several brands of agar at 0.7-0.8% are widely used as gelling agents (MURASHIGE 1977; JAIN and BABBAR 2002). Gellan gum (Gelrite) at 0.2-0.3% is also used although hyperhydricity of shoots in some species has been attributed to low concentrations of Gelrite (JAIN and BABBAR 2002). Use of liquid medium is cost effective and favours fast growth. However, shoots grown in a liquid medium may become hyperhydric if they are submerged (GEORGE 1993).

The addition of substances such as activated charcoal (AC) may influence the physiological response of tissues in culture either positively or negatively (VAN WAES 1987). Addition of charcoal in the nutrient media reduced shoot chlorosis and leaf necrosis in *Cercis canadensis* var *mexicana* (MACKAY *et al.* 1995) but failed to control hyperhydricity in *Rhanterium apapposum* (ABO EL-NIL 1997). Applicability of charcoal in tissue culture is based on its adsorptive properties of undesired, excess or toxic substances, provision of a dark matrix, improving aeration but may also adsorb essential PGRs and other organic compounds (FRIDBORG and ERIKSSON 1975; FRIDBORG *et al.* 1978).

Light and temperature are the major physical requirements of cells/tissues in culture (COLLINS and EDWARDS 1998). Light intensity of about 1,000-3,000 lux and a photoperiod of 16/8 hours are optimal for most plant species (MURASHIGE 1977). However, plant species that are prone to production of phenolic substances benefit from placement in the dark for a few days before they are exposed to lighted conditions (DHAR and UPRETI 1999). Temperature should to a large extent simulate that of the

natural habitat (MURASHIGE and HUANG 1987). However, 25±2 °C is optimal for most plant species (GEORGE 1996). In *Citrus chinensis,* 27 °C was found to be the optimal temperature for shoot initiation and multiplication (DURAN-VILA *et al.* 1992). In *Aloe polyphylla*, there was no significant difference in shoot production in media supplemented with kinetin at 20 °C or 25 °C although temperatures below or above this range led to a reduction in the number of shoots produced per explant (CHUKWUJEKWU *et al.* 2002).

2.6.1.2 Acclimatization

Rapid mass production of uniform plantlets that can successfully establish in the field is the ultimate goal of *in vitro* plant propagation (BATLLE and ALDRUFEU 1987; SHORT *et al.* 1987), hence this stage is very important as it can lead to significant losses (SEABROOK 1980; GEORGE 1996). Since the plantlets are grown under heterotrophic conditions, they should gradually be adapted to convert to an autotrophic state (MURASHIGE 1974).

Placement of the plantlets in an environment of very high humidity minimizes water loss. This is because the waxy cuticle and stomatal openings in the leaves of *in vitro* produced shoots are usually inadequately formed (ALDRUFEU 1987; GEORGE 1996). Anatomical studies on the leaves of *in vitro* grown *Litsea cubeba* shoots revealed that the cuticle and the guard cells were imperfectly formed. Such plantlets had very low survival rates when directly transplanted to the nursery (MAO *et al.* 2000). A good root system at this stage also helps to minimize some moisture stress (SEABROOK 1980). However, the roots produced *in vitro* are not very efficient at water absorption (GEORGE 1996) and hence the plants should be placed under shade until efficient roots are produced. The plants should be transferred to a green house before they are finally propagated in the field to ensure that they are sufficiently adapted to field conditions (PIERIK 1987).

2.6.2 Tissue culture of *Maytenus* species

Several *Maytenus* species especially those used in traditional medicine (Table 1.1) have been subjected to tissue culture studies. However, there are no reports on tissue culture studies on *Maytenus senegalensis*.

Apical meristems obtained from new growth of *M. canariensis* were induced to produce multiple shoots in Woody Plant Medium (WPM) supplemented with MS micronutrients, BA, IAA, 15µM potassium nitrate (KNO_3) and gibberellic acid. The shoots were rooted *in vitro* in quarter strength WMP medium supplemented with a combination of IAA and NAA (MENDEROS-MOLINA 2002). *M. emarginata* nodal explants produced axillary shoots when placed in MS salts supplemented with BA (2.5 mgl^{-1}) and IAA (0.1 mgl^{-1}). Rooting was best when the shoots were placed on half-strength MS liquid medium supplemented with 25 mgl^{-1} IBA in the dark for 72 h (RATHORE *et al.* 1992).

Shoot initiation and multiplication in *M. ilicifolia* axillary buds was achieved in the presence of MS salts supplemented with BA. Addition of low concentrations of IAA promoted shoot lengths but not the number of shoots produced per explant. Rooting was accomplished *ex vitro* without an addition of exogenous auxins (PEREIRA *et al.* 1995). Induction of callus formation for production of bioactive secondary products has been achieved in *M. aquifolium* (FRANCA *et al.* 1999), *M. ilicifolia* (PEREIRA *et al.* 1993) and *M. buchananii* (KUTNEY *et al.* 1991).

2.6.3 *In vitro* root culture

Most medicinal natural products continue to be extracted from plants (ALFERMANN and PETERSEN 1995). However, most of these plants are grown or collected from the wild in the tropics (PUSHPANGADAN and THOMAS 1999) while the production of pharmaceuticals is done in temperate countries. The habitats in which most of these plants are found are being destroyed at very fast rates (AKETCH 1992; ETKIN 1998). World-wide resurgence of interest on natural products of plant origin in particular have l

led to overexploitation of some medicinal plant species (HEYWOOD 1999). This scenario implies that problems of inconsistent supplies are eminent (ALFERMANN and PETERSEN 1995).

Plant cells in various forms including suspended cells, immobilised cells and organs such as roots and shoots can be used for *in vitro* production of valuable secondary products. However, the concentration of the active secondary metabolites is usually very low in cell suspension cultures and the tendency to exhibit genetic instability is high (SHIMOMURA and KITAZAWA 1991).

Hairy roots, genetically transformed using *Agrobacterium* spp, are currently extensively being studied as potential sources of secondary metabolites (SHANKS and MORGAN 1999). They have the advantages of being genetically stable, have rapid growth rates, are high yielding and can grow without the addition of Plant Growth Regulators (PGRs) (KUSIKARI *et al.* 2000). However, this technique has limitations in that its application is limited to some genera. Attempts to transform the roots of *Bupleurum falcatum* were unsuccessful (KUSIKARI *et al.* 2000). Transformation frequency may also be very low as in *Solanum mauritianum* (DREWES and VAN STADEN 1995).

Adventitious *in vitro* root cultures can be used as alternative sources of secondary metabolites (DODDS and ROBERTS 1985). Derivatives of tropane alkaloids, which have pharmaceutical applications, have been produced in root cultures of *Atropa, Datura* and *Duboisia* species (HASHIMOTO and YAMANDA 1986). Gluchochidiol and gluchidones, compounds with anti-inflammatory properties, have been isolated from *Phyllanthus carolinensis* root cultures (CATAPAN *et al.* 2000). Cell suspension cultures may fail to produce the required secondary metabolites as a result of a differentiation requirement. In *Datura stramonium* for instance, differentiated root cultures produced higher levels of tropane alkaloids than the cell suspension cultures (MALDONADO and MENDOZA 1992).

Some root cultures exhibit high levels of secondary metabolites production. In *Salvia miltiorrhiza*, the production of tanshinones was six times more than that of the mother plant (SHIMOMURA and KITAZAWA 1991). A *Boerhaaria diffusa* root culture yielded higher levels of punarnavosid than the field grown plants (PATHAK *et al.* 1998).
Root cultures can facilitate uniform production of secondary metabolites. In some plant species, variations in growth and morphological characteristics may arise as a result of genetic and environmental factors thereby influencing the production of some secondary metabolites. *Bupleurum falcatum* exhibit growth and morphological variations, which influence the production of saikosaponins (KUSAKARI *et al.* 2000).

Growth and secondary metabolites production in root cultures is influenced by factors such as media type and strength (AHÉÉ and DUHOUX 1994), light (TANIGUCHI *et al.* 2000), carbohydrate sources and concentration (MARÍN and MARÍN 1996) and PGRs (HASHIMOTO and YAMANDA 1986) among others.

CHAPTER 3

ANTIBACTERIAL AND ANTI-INFLAMMATORY ACTIVITIES OF SOME PLANTS USED FOR MEDICINAL PURPOSES IN KENYA

3.1 INTRODUCTION

Infections associated with bacterial pathogens and inflammatory diseases are among some of the ailments that are treated using remedies prepared from various plant species in Kenya (Table 3.1). There is need to validate the biological activities of these plants as a tool to facilitate the official integration of traditional medicine with modern medicine (JÄGER *et al.* 1996). The full potential of higher plants as sources of new drugs is still largely unexplored (BALANDRIN *et al.* 1993; FARNSWORTH 1994). Validation of efficacy thus forms a good basis for selection of plants warranting further pharmacological studies for drug discovery. It is also a good justification for conservation efforts on medicinal plants.

Biologically active components may be missed because of insensitivity of *in vitro* assays (PRANCE 1994). Availability of suitable bioassays is thus crucial to an investigation of plants for biological activity. The bioassays can either be *in vitro* or *in vivo* (SUFFNESS and DOUROS 1982). For a bioassay to be effective, it should be simple, rapid, and reproducible as well as cost effective and sensitive (McCHESNEY 1993). Many current *in vitro* bioassays possess these attributes and provide important preliminary information for the evaluation of therapeutic potential of plants (McCHESNEY 1993). Nevertheless, it should be borne in mind that in an *in vitro* bioassay, only *in vitro* data is provided and activity may not correspond to *in vivo* activity (SUFFNESS and DOUROS 1982). However, due to advancement in the type of bioassays, a plant that tested negative in a screen 20 years ago, would not necessarily give a negative result today (PRANCE 1994).

3.1.1 Antibacterial agents

Although it is widely acknowledged that bacterial infections are among the few diseases in medicine in which specific therapy is available (SLEIGH and TIMBURY 1998), there is a need to develop new, more effective and less toxic antibiotics (CLARK and HUFFORD 1993). This is prompted by emergence of drug-resistant bacterial strains (RUSSEL and CHOPRA 1996; SLEIGH and TIMBURY 1998), significant toxicities and failure rates especially in treatment of bacterial infections associated with HIV/AIDS (CLARK and HUFFORD 1993). Bacterial infections are also still common especially in the developing countries due to poor and unhygienic living conditions (RASOANAIVO and RATSIMAMANGA-URVEG 1993). Besides, most of the inhabitants of the developing countries cannot access and/or afford conventional antibiotics (IWU 1994). Standardized antibacterial plant extracts would alleviate the problem of cost, especially in the developing countries (FARNSWORTH *et al.* 1985).

On the basis of their site of action (GREENWOOD 1992; LAUGHLIN and FAIRFIELD 1997; MURRAY *et al.* 1997), antibacterial agents can be categorized into groups, which include:

(i) Inhibitors of cell wall synthesis;
(ii) Inhibitors of bacteria protein synthesis; and
(iii) Inhibitors of nucleic acid synthesis.

Microorganisms have been the sources of antibiotics in the past (EVANS 1996). However, higher plants are potential sources of prototype drugs with different mechanisms and less likelihood of toxicity and resistance (CLARK and HUFFORD 1993; LAUGHLIN and FAIRFIELD 1997; COWAN 1999). Several groups of higher plant secondary products such as phenols, flavones, quinones, coumarins, alkaloids, terpenoids and essential oils (COWAN 1999) and fatty acids (McGAW *et al.* 2002) exhibit antibacterial activity. Any part of the plant may contain active components. For instance, fatty acids in the leaves of *Schotia brachypetala* (McGAW *et al.* 2002) and muzigadial, in the stem-bark of *Warbugia salutaris* (RABE and VAN STADEN 2000).

In vitro antibacterial assays can be classified into three groups, namely diffusion, dilution and bioautographic methods (COWAN 1999; TAYLOR *et al.* 2001). In screening plant extracts against a wide range of bacterial strains, diffusion and dilution methods are commonly used (COWAN 1999; TAYLOR *et al.* 2001).

Diffusion assays involves spreading of a suspension of the test bacterial strain over adjacent parts of an agar plate. The antibacterial agents diffuse from the agar wells or discs into the medium, killing (bactericidal) or inhibiting the growth of organisms sensitive to them (bactreostatic) (BURTON and ENGELKIRK 1995; INGLIS 1997). The zone of inhibition can then be measured (GREENWOOD 1992; INGLIS 1997). This bioassay is advantageous in that it is rapid, sensitive and simple but effective in assessing the presence or absence of desired antibacterial activity (CLARK and HUFFORD 1993). Nevertheless, it is limited in that large molecule compounds may produce small zones due to slow diffusion in agar (INGLIS 1997).

In the dilution assay, plant extracts are incorporated into seeded wells and serially diluted. The Minimum Inhibitory Concentration (MIC), which is the lowest concentration that inhibits the growth of bacteria is then determined (MURRAY *et al.* 1992; COWAN 1999). In the agar overlay method, Thin Layer Chromatography (TLC) is combined with a bioassay *in situ* and facilitates localization of active components (HAMBURGER AND CORDELL 1987).

3.1.2 Cyclooxygenase inhibition

Inflammation is the response of tissues to injury (VANE and BOTTING 1996). It involves a complex battery of enzyme activation, mediator release, extravasation of fluid, cell migration, tissue breakdown and repair (VANE and BOTTING 1996). The biosynthesis of prostaglandins, which act synergistically with other mediators to produce inflammatory pain, is catalyzed by prostaglandin H_2 synthase (PGH_2) or cyclooxygenase (COX) (VANE 1994; VANE and BOTTING 1996). Modulation of the activity of the enzyme implies that the inflammation process can be modified.

COX is multi-functional enzyme, incorporating both cyclooxygenase and peroxidase activity. It exists in two isoforms, COX-1, which is constitutive, and COX-2, which is induced in a number of cells by pro-inflammatory stimuli (VANE 1994; VANE and BOTTING 1996).

Non-steroidal anti-inflammatory drugs (NSAIDS) inhibit COX enzymes both *in vitro* and *in vivo* (WEEKS 1973; VANE 1994; VANE and BOTTING 1996). Unfortunately some of the very effective NSAIDS like aspirin and indomethacin are associated with unwanted side effects such as irritation of the stomach lining and toxic effects on the kidney. Such NSAIDS are more potent inhibitors of COX-1 than COX-2 (VANE 1994; VANE and BOTTING 1996). COX-1 is responsible for the production of prostanoids that maintain mucosal blood flow, promote mucosal secretion, inhibit neutrophil adherence and maintain renal blood flow (TAYLOR and VAN STADEN 2001). Prostanoids produced by COX-2 are involved in the inflammatory process and promote oedema, fever and pain (TAYLOR *et al.* 2001). Inhibition of COX-2 is not associated with unwanted side effects. Consequently, the recent past has witnessed an intensive search for compounds that selectively inhibit COX-2 (VANE 1994; VANE and BOTTING 1996).

There are reports in ethnomedical literature that medicinal plants are traditionally used in treatment of conditions/ailments associated with inflammation and pain such as rheumatism (Table 3.1).

Drugs for the control of symptoms such as pain can be obtained from plants (TYLER 1986). Already, promising NSAIDS have been found in various groups of secondary products obtained from higher plants such as flavonoids, napthraquinones, tannins, simple phenols, alkylamides and thiosulphinates (TAYLOR and VAN STADEN 2001). Screening plant species with folkloric uses not only provides a rationale for their use in traditional medicine but also increases the chances of finding potent therapeutic compounds (COTTON 1996).

3.2 MATERIALS AND METHODS

3.2.1 Ethnomedical information and plant collection

The criteria for selection of plant species tested in this study was based upon their use in treatment of infections associated with bacterial pathogens and inflammatory diseases by Kenyan traditional healers as reported in literature (Table 3.1). Plant materials were collected from natural populations in the Central and Eastern provinces of Kenya, areas lying within the vicinity of Nairobi. The botanical identification of the collected plant species was confirmed by Mr Onesmus Mwangangi and Mr Joshua Muasya of the East African Herbarium, Nairobi, Kenya. Voucher specimens were deposited in this Herbarium. The voucher specimen numbers of the plants collected are given in Table 3.1. Although *Galinsoga parviflora* Cav. is not indigenous to Kenya, it is cosmopolitan in distribution and its use in traditional medicine is widely reported.

Table 3.1 Selected Kenyan medicinal plants investigated for antibacterial and anti-inflammatory activity.

Plant species (family)	Voucher specimen No.	Traditional medicinal uses (KOKWARO 1976; GACHATHI 1989 ; BEENTJE 1994 ; MAUNDU *et al.* 1999).
Ajuga remota Benth (Lamiaceae)	ENM 012EA	Aerial parts are used for treatment of malaria, toothache, dysentery, liver problems and high blood pressure.
Conyza schimperiana A. Rich (Asteraceae)	ENM 04EA	Roots are used for treatment of headaches, tonsillitis and influenza. Leaves are used for treating malaria and indigestion.
Croton macrostachyus Del. (Euphorbiaceae)	ENM 09EA	Roots are used as anthelmintics and for treatment of malaria and venereal diseases. The bark is used to treat skin rash in newborn babies. Leaves are used to treat coughs and leaf juice improves blood clotting.
Croton megalocarpus Hutch (Euphorbiaceae)	ENM 010EA	Bark is used as anthelmintics and to treat whooping cough, severe colds and pneumonia.
Galinsoga parviflora Cav. (Asteraceae)	ENM 011EA	The stems and the leaves are used to treat colds and sores.

Table 3.1 contd.

Plant species (family)	Voucher specimen No.	Traditional medicinal uses (KOKWARO 1976; GACHATHI 1989 ; BEENTJE 1994 ; MAUNDU *et al.* 1999).
Maytenus senegalensis (Lam.) Exell (Celastraceae)	ENM 01EA	Roots are used in treatment of chest pains, rheumatism, snakebites, diarrhea and fever. Leaves are used in treating eye infections.
Mondia whitei (Hook F.) Skeels (Periplocaceae)	ENM 07EA	Roots are used for treating gonorrhea, as an aphrodisiac and for stomach disorders.
Oxygonum sinuatum (Meisn.) Damn (Polygonaceae)	ENM 08EA	Roots are used for bilharzia. Leaf juice is used in treatment of conjunctivitis while stems are used to treat tonsillitis.
Plectranthus barbatus Benth (Lamiaceae)	ENM 06EA	Roots are used for treatment of chest troubles. Leaves are used to treat stomachache, measles and as a purgative.
Spiranthes mauritianum (Pers.) D.C. (Asteraceae)	ENM 05EA	Roots are used to treat sores in the mouth, gums and throat. Flower heads are chewed to relief toothache.

Table 3.1 Contd

Plant species (family)	Voucher specimen No.	Traditional medicinal uses (KOKWARO 1976; GACHATHI 1989 ; BEENTJE 1994 ; MAUNDU *et al.* 1999).
Zanthoxylum chalybeum Engl. (Rutaceae)	ENM 02EA	Barks or roots are used for treating malaria, colds, coughs, toothache, sores wounds and headache. Fruits can also be used in treatment of coughs while leaves are used in treating severe colds and pneumonia.
Zanthoxylum usambarense (Engl.) Kokwaro (Rutaceae)	ENM 03EA	Bark is used to treat rheumatism. Young twigs are used as toothbrushes.

3.2.2 Preparation of extracts

Plant material used in the screening was dried at room temperature for a period of two weeks and then ground into fine powders. The powders were stored in sealed airtight bags in the dark at room temperature until extraction. Three solvents, n-hexane (non-polar), methanol (moderately polar) and water (polar) were used for extraction. For the methanol and water extracts, 2 g of plant material was extracted with 20 ml of the solvent while for hexane extracts, 4 g of plant material was extracted with 40 ml hexane. Extraction was done by sonication for 30 min in an ultra sound bath (Branson 2210, 47 kHz) after which the plant extracts were macerated overnight, then filtered using Whatman No. 1 filter paper. The clear filtrates were placed under a stream of air to dry.

For the antibacterial assay, the residues were resuspended (redissolved) in their extracting solvents to give 100 mg residue ml^{-1}. For the anti-inflammatory bioassay, the organic extracts were resuspended in ethanol while the aqueous extracts were resuspended in water to give 20 mg residue ml^{-1} and 2.5 mg residue ml^{-1} respectively.

3.2.3 Antibacterial activity

The disc-diffusion assay (RASOANAIVO and RATSIMAMANGA-URVEG, 1993) was used to determine the growth inhibition caused by plant extracts against the following bacterial strains: *Bacillus subtilis* (ATCC 6150), *Micrococcus luteus* (ATCC, 4698), *Staphylococcus aureus* (ATCC 12600) (Gram-positive), *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883) (Gram-negative). Bacteria were maintained at 4 °C on nutrient agar (NA) plates.

Base plates were prepared by pouring 10 ml Mueller-Hinton (MH) agar into sterile Petri dishes (9 cm) and allowed to set. Molten MH agar held at 48 °C was inoculated with a broth culture (10^6-10^8 bacteria ml^{-1}) of the test organism and poured over the base plates forming a homogenous top layer. Filter paper discs (Whatman No.3, 6 mm diameter) were sterilized by autoclaving. Ten µl of plant extract (100 $mgml^{-1}$) were applied per filter paper disc so that each disc contained 1 mg of material. The discs were air-dried and placed onto the seeded top layer of the MH agar plates. Each extract was tested in quadruplicate (4 discs per plate), with a neomycin (500 μgml^{-1}) disc as reference or positive control. Air-dried solvent (hexane, methanol and water) saturated discs were used as negative controls. The plates were evaluated after incubation at 37 °C for 18 h after which the zones of inhibition around each disc were measured.

The ratio of the inhibition zone (mm) produced by the plant extract and the inhibition zone around the neomycin reference (mm) was used to express antibacterial activity (VLIËNTINCK *et al.* 1995). The activity of neomycin was included in this equation to adjust for plate-to-plate variations in the sensitivity of a particular bacterial strain (RABE and VAN STADEN, 1997). Results given are the mean (±SE) of two experiments.

3.2.4 Anti-inflammatory activity

Anti-inflammatory activity was tested using the cyclooxygenase assay (COX-1). The assay was performed as described by WHITE and GLASSMANN (1974) with slight modifications (JÄGER *et al.* 1996). Cyclooxygenase enzyme was prepared from sheep seminal vesicle microsomal fractions. Cyclooxygenase enzyme solution and co-factor solution (0.3 $mgml^{-1}$ adrenalin and 0.3 $mgml^{-1}$ reduced glutathione in 0.1 M Tris buffer, pH 8.2) were mixed in a ratio of 1:5 and incubated on ice for 15 min. Sixty microlitres of this enzyme/co-factor solution was added to 20 µl of sample (2.5 µl of ethanolic extract and 17.5 µl water; 20 µl aqueous extract) and incubated at room temperature for 5 min. Twenty microlitres of ^{14}C-arachidonic acid were added and the assay mixture incubated for 10 min at 37 °C. The reaction was terminated by adding 10 µl of 2M HCl. After incubation, 4 µl of unlabelled prostaglandins (PGE_2:PGF2 1:1; v/v) were added as a carrier solution.

The ^{14}C-labelled prostaglandins synthesized during the assay were separated from the unmetabolized ^{14}C-arachidonic acid by column chromatography. Silica gel in eluent 1 (hexane: dioxan: acetic acid 350:150:1; v/v/v) was packed in Pasteur pipettes to a height of 3 cm. One ml of eluent 1 was added to each of the assay mixtures and this mixture was applied to separate columns. The arachidonic acid was eluted with 4 ml of eluent 1 and discarded. Labelled prostaglandins were eluted with 3 ml of eluent 2 (ethylacetate: methanol (85:15; v/v) into scintillation vials. Scintillation fluid (4 ml) was added and the radioactivity counted after 30 min in the dark using a Beckman LS6000LL scintillation counter. Extracts were tested at a concentration of 500 μgml^{-1} with double determinations for each extract per assay. Percentage inhibition of test solutions was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank (2.5 µl ethanol and 17.5 µl water). Positive control measurements were carried out with indomethacin solution (20 mM). The results given are the mean (±SE) percentages of two experiments (% inhibition).

3.3 RESULTS AND DISCUSSION

3.3.1 Antibacterial activity

The antibacterial activity of crude extracts (hexane, methanol and water extracts at a concentration of 100 $mgml^{-1}$) showing positive results is presented in Table 3.2. Plant species exhibiting no antibacterial activity were: *Ajuga remota*, *Oxygonum sinuatum* and *Mondia whitei*. VLIETINCK *et al.* (1995) also reported the absence of antibacterial activity in extracts of *Oxygonum sinuatum* collected in Rwanda.

Activity was only detected against Gram-positive bacterial strains (Table 3.2). No bactericidal activity (indicated by the presence of a clear zone around the disc) was shown against Gram-negative bacteria with the exception of bacteriostatic activity (indicated by a translucent zone around the disc, which implies that the growth of the initial bacterial cells was only inhibited but they were not killed) by some extracts against *K. pneumoniae* (results not shown). Permeation of the cell wall by drugs is more complex in Gram-negative bacterial strains (BROWN *et al.* 1979). Consequently, various workers have reported most antibacterial activity against Gram-positive bacterial strains (VLIETINCK *et al.*, 1995, RABE and VAN STADEN 1997 and RABE and VAN STADEN 1998). *Staphylococcus aureus* was the most susceptible bacterial strain.

The stem-bark and the root-bark extracts of *Maytenus senegalensis* exhibited activity with the methanolic extract of the stem-bark exhibiting the highest activity overall, 1.68±0.01 against *Staphylococcus aureus*. The water extract of the stem-bark also had activity with a value of 1.03±0.27. Methanolic and water extracts of the root bark yielded anti-bacterial activity with values of 1.18±0.17 and 0.7±0.07 respectively.

Table 3.2.Antibacterial activity[a] displayed by hexane, methanol and water extracts (100 mgml^{-1}) of some plants used for medicinal purposes in Kenya.

Plant species	Plant part	Extract	[b]Bacterial strains		
			B.s	*M.l*	*S.a*
C. schimperiana	[c]Aerial parts	Hexane	0.00	0.00	0.00
		Methanol	0.10±0.00	0.00	0.80±0.01
		Water	0.00	0.00	0.00
	Roots	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00
C. macrostachyus	Roots	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00
	Stem-bark	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00
	Leaves	Hexane	0.19±0.00	0.08±0.01	0.08±0.01
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00
C. megalocarpus	Roots	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00
	Stem-bark	Hexane	0.14±0.00	0.00	0.00
		Methanol	0.12±0.12	0.00	0.13±0.00
		Water	0.00	0.00	0.00
	Leaves	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00

Table 3.2 contd.

Plant species	Plant part	Extract	Bacterial strains		
			B.s	*M.l*	*S.a*
G. parviflora	Aerial parts	Hexane	0.28±0.02	0.14±0.00	0.88±0.01
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00
M. senegalensis		Hexane	0.00	0.00	0.00
	Root-bark	Methanol	0.18±0.02	0.13±0.01	1.18±0.17
		Water	0.18±0.01	0.00	0.70±0.07
	Stem-bark	Hexane	0.00	0.00	0.00
		Methanol	0.19±0.04	0.15±0.02	1.68±0.01
		Water	0.22±0.02	0.17±0.01	1.03±0.27
	Leaves	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00
P. barbatus	Roots	Hexane	0.14±0.01	0.17±0.03	0.39±0.01
		Methanol	0.23±0.01	0.44±0.00	0.95±0.04
		Water	0.00	0.00	0.00
	Stem	Hexane	0.09±0.01	0.12±0.08	0.32±0.01
		Methanol	0.00	0.26±0.00	0.00
		Water	0.00	0.25±0.01	0.00
	Leaves	Hexane	[d]ND	ND	ND
		Methanol	0.47±0.01	0.18±0.00	0.89±0.02
		Water	0.00	0.00	0.00
S. mauritianum	Whole plant	Hexane	0.00	0.00	1.03±0.03
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00

Table 3.2 contd.

Plant species	Plant part	Extract	Bacterial strains		
			B.s	*M.l*	*S.a.*
Z. chalybeum	Root-bark	Hexane	0.38±0.01	0.00	0.70±0.07
		Methanol	0.53±0.07	0.87±0.01	1.00±0.14
		Water	0.00	0.00	0.00
	Stem-bark	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.29±0.01	0.00
		Water	0.00	0.00	0.00
	Leaves	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00
Z. usambarense	Root-bark	Hexane	0.19±0.00	0.00	0.00
		Methanol	0.08±0.01	0.00	1.09±0.01
		Water	0.58±0.01	0.00	0.41±0.01
	Stem-bark	Hexane	0.00	0.17±0.03	0.00
		Methanol	0.40±0.01	0.00	0.30±0.01
		Water	0.00	0.00	0.00
	Leaves	Hexane	0.00	0.00	0.00
		Methanol	0.00	0.00	0.00
		Water	0.00	0.00	0.00

[a]The antibacterial activity is expressed as the ratio of the inhibition zone of the extract (100 $mgml^{-1}$) to the inhibition zone of the reference (neomycin, 500 μgml^{-1}). Values are means ±SE of results obtained from two experiments.

[b]*B.s.*,*Bacillus subtilis*; *M.l.*, *Micrococcus luteus* ;*S.a.*, *Staphylococcus aureus*

[c]Whole plant minus the roots.

[d]Not determined.

Antibacterial compounds such as phenol nor-terpene (GONZÁLEZ *et al.* 1996), norterpene quinone methides (GONZÁLEZ *et al.* 1998; ALVARENGA *et al.* 1999) have been isolated from other *Maytenus* species. There are possibilities that similar or related compounds are present in the stem-bark and root barks of *Maytenus senegalensis*, which could be responsible for the high antibacterial activity observed.

The roots, stem-bark and the leaves of *Plectranthus barbatus* exhibited activity. Leaves of members of the family Lamiaceae are known to contain terpenoids, which possess antifungal, antibacterial and anti-insect activities (COLE 1992). Leaf extracts of some South African *Plectranthus* species showed antibacterial activity (RABE and VAN STADEN 1998). Presence of activity in the leaf extracts implies that there could be the possibility of substituting leaves for roots and stems when utilizing this plant species for treatment of bacterial related infections. Harvesting of leaves for medicinal purposes is more sustainable compared to harvesting of plant parts such as roots and stems, which if over harvested could threaten the survival of the plant (ZSCHOCKE *et al.* 2000).

The root and stem bark extracts of the two *Zanthoxylum* species showed high antibacterial activity. Antibacterial activity was reported for the bark extracts of *Fagara chalybea* (synonymn of Z. *chalybeum*) (TANIGUCHI and KUBO, 1993) with the activity being attributed to two alkaloids: N-methylfindesine and flindersine. Cherelythrine, an alkaloid with antibacterial activity was isolated from *Z. chalybeum* (ADDAE-MENSAH 1992) and *Z. clava-herculis* (GIBBONS *et al.* 2003).

With the exception of the hexane extracts of the leaves of *Croton macrostachyus*, the extracts of *C. macrostachyus* and *C. megalocarpus*, exhibited low antibacterial activity. Antibacterial activity was previously detected in the bark of *C. macrostachyus* collected from East Africa (TANIGUCHI and KUBO, 1993). This study could not confirm these previous results. The stem-bark extracts of *C. megalocarpus* exhibited low activity. Earlier studies (TANIGUCHI and KUBO 1993) reported the absence of antibacterial activity from bark extracts of *C. megalocarpus*. The therapeutic potency of biologically active components in plants can be affected by locality of the plant species and time of

collection among other factors (PRANCE 1994). These factors could be responsible for the contrasting observations to some extent.

The hexane extract of the aerial parts of *Spiranthes mauritianum* showed high activity (1.03±0.03) against *S. aureus*. This observation confirms the work of FABRY *et al.* (1998) who reported antibacterial activity in the roots and flowers of *S. mauritianum*. High antibacterial activity was also shown by the hexane extract of *Galinsoga parviflora* and the methanolic extract of *Conyza schimperiana* aerial parts.

3.3.2 Anti-inflammatory activity

The results obtained for extracts in the cyclooxygenase (COX-1) assay at a test concentration of 500 μgml^{-1} are given in Table 3.3. The indomethacin control (20 mM) inhibited cyclooxygenase to a level of 76±4.5%. In order to classify a plant as active, the criteria used by JÄGER *et al.* (1996) was followed. Following these criteria, the minimum inhibition should be 50 and 70% for aqueous and organic extracts respectively, at a test concentration of 500 μgml^{-1}. This minimum inhibition criterion is useful when selecting plant species warranting further studies.

Table 3.3 The percentage inhibition of cyclooxygenase exhibited by extracts (500 µgml^{-1}) of some plants used for medicinal purposes in Kenya.

Plant species	Plant part	[a]Inhibition (%)		
		Hexane	Methanol	Water
A. remota	[b]Aerial parts	90.3±0.8	74.0±0.1	47.0±2.0
C. schimperiana	Roots	47.0±2.5	65.0±4.5	16.0±2.0
	Aerial parts	69.0±5.5	87.0±3.5	27.0±4.0
C. macrostachyus	Roots	37.0±05	65.0±3.0	40.0±1.5
	Stem-bark	94.0±2.5	97.0±1.8	79.0±1.1
	Leaves	90.6±1.8	85.0±3.5	74.0±2.5
C. megalocarpus	Roots	70.0±0.3	81.0±1.4	29.0±4.0
	Stem-bark	68.0±4.5	34.0±1.0	4.0±2.0
	Leaves	79.0±0.3	69.0±1.0	65.0±3.0
G. parviflora	Aerial parts	68.0±4.5	90.0±1.5	54.0±2.5
M. senegalensis	Root-bark	49.0±2.0	93.0±1.0	84.0±2.5
	Stem-bark	40.0±0.8	81.0±1.0	53.0±2.0
	Leaves	32.0±1.9	67.0±0.7	21.0±0.6
M. whitei	Roots	85.0±1.3	93.0±2.0	48.0±5.5
	Leaves	51.0±2.5	93.0±4.0	67.0±2.0
O. sinuatum	Aerial parts	91.0±0.1	91.0±1.9	42.0±1.8
P. barbatus	Roots	93.8±0.1	95.6±0.6	34.0±2.5
	Stem	70.0±1.4	87.0±4.5	10.0±1.0
	Leaves	[c]ND	88.0±3.5	7.0±1.5

Table 3.3 Contd.

Plant species	Plant part	Inhibition (%)		
		Hexane	Methanol	Water
S. mauritianum	Whole plant	76.0±2.0	84.0±2.0	32.0±3.0
Z. chalybeum	Root-bark	67.0±1.5	94.8±2.4	73.7±1.4
	Stem-bark	57.0±1.0	89.0±2.3	56.0±1.5
	Leaves	58.0±3.0	87.0±3.0	30.0±0.5
Z. usambarense	Root-bark	86.0±2.0	99.0±1.5	78.0±1.4
	Stem-bark	97.5±2.8	87.5±3.8	64.5±2.9
	Leaves	84.6±1.4	92.8±2.6	46.0±2.5

[a] Values are the mean percentages (±SE) of results obtained from two experiments.

[b] Whole plant minus the roots.

[c] Not determined.

The results obtained from this study indicated that all the plant species tested could be classified as active (Table 3.3). The highest inhibition was with organic extracts of *Ajuga remota*, *Croton macrostachyus*, *Galinsoga parviflora*, *Maytenus senegalensis*, *Mondia whitei*, *Oxygonum sinuatum*, *Plectranthus barbatus*, *Zanthoxylum chalybem* and *Zanthoxylum usambarense*. Generally water extracts showed lower activity except in the case of *Croton macrostachyus*, *Maytenus senegalensis, Zanthoxylum chalybem* and *Zanthoxylum usambarense*, which showed inhibitory activity of over 70%. This observation is interesting because water is the solvent that is mostly used by Traditional Medical Practitioners in preparing traditional remedies in the form of infusions, decoctions, concoctions or poultices. However, considering that the dosage dispensed by the traditional doctors is usually very high, for instance three to four cups per day for an adult (SHALE *et al.* 1999), water can then still be considered as an appropriate extracting solvent for traditional medicinal plant remedies.

Among the plants investigated in this study, the only plant species that has been investigated with respect to anti-inflammatory activity is *Z. chalybeum*. MULLER-JAKIC *et al.* (1993) identified protoberberines and benzophenanthridine type of alkaloids in the bark, which were found to have anti-inflammatory activities. Z. *chalybeum* and *Z. usambarense* (KATO *et al.* 1996) contain similar alkaloid profiles. It is thus likely that alkaloids with anti-inflammatory activity occur in *Z. usambarense* as well and are responsible for the high anti-inflammatory activity observed.

Croton megalocarpus extracts only inhibited cyclooxygenase to a moderate extent. A similar observation was made on the bark of *Croton silvaticus* (JÄGER *et al.* 1996). However, *C. macrostachyus* extracts yielded very high levels of inhibition. Anti-inflammatory activity has been reported in *C. cajucara*. Two diterpenes: cajucarinolide and isocajucarinolide were found to inhibit phosholipase A_2 *in vitro*, an early enzyme in the archidonate pathway (ICHIHARA *et al.* 1991).

3.4 CONCLUSIONS

In conclusion, the results obtained in this part of the study confirmed the therapeutic potency of some of the twelve plant species analysed and thus provide a rationale for their use in traditional medicine. These results form a good basis for selection of candidate plant species for further pharmacological and toxicity studies and for conservation purposes. However, there is need to screen these plant species for anti-inflammatory activity against COX-2 enzyme. Plant species exhibiting higher inhibition of COX-2 compared to COX-1 are likely to yield anti-inflammatory agents with minimal unwanted side effects. The kind of screening was not undertaken in this study due to unavailability of plant materials.

CHAPTER 4

MICROPROPAGATION OF *MAYTENUS SENEGALENSIS* (Lam.) Exell

4.1 INTRODUCTION

The world's botanical resources are being depleted at very fast rates (ABO EL-NIL 1997; ETKIN 1998). The rate is particularly high in developing countries. Since the later half of the 20th century, there has been exponential population growth rates in these countries, leading to increased demand for plant resources and destruction of their habitats to pave way for agricultural and settlement land among other development activities (WOCHOK 1981; ETKIN 1998; JÄGER and VAN STADEN 2000). Estimates by the International Union for Conservation of Nature (IUCN) and the World Wildlife Fund (WWF) indicate that up to 60,000 higher plant species could become extinct or nearly extinct by the year 2050 if the current trends continue (ETKIN 1998).

In addition to their contribution to the integrity of the environment (HARRY and THORPE 1994), plants are also invaluable sources of therapeutic agents among other uses. Traditional medicine, which is the major mode of health care in the rural parts of developing countries, is mostly plant based (JÄGER and VAN STADEN 2000). Apparently most of the plants employed in traditional medicine are collected from the wild. It is therefore not surprising to find reports that the populations of some medicinal plants are on the decline (MARSHALL 1998). Efforts to conserve the affected plant species should thus be made to preserve them for future generations (BALANDRIN and KLOCKE 1988).

Sustainable utilization of medicinal plants is one of the potential conservation options. However, its applicability is limited due to the persistent high demand (VAN STADEN 1999). Cultivation of medicinal plants on the other hand would alleviate pressure from

wild sources and also provide a source of livelihood for the unemployed commercial medicinal plant gatherers as well (VAN STADEN 1999; JÄGER and VAN STADEN 2000). However, cultivation of medicinal plants in African countries accounts for a very small percentage (KOKWARO 1993) and only medicinal plants that are sold in international markets have been put under cultivation (JÄGER and VAN STADEN 2000).

Maytenus senegalensis extracts are widely used in traditional medicine (Table 1.1) and they were found to exhibit antibacterial (Table 3.2) and anti-inflammatory activities (Table 3.3). However, the populations of *M. senegalensis* are on the decline in east Africa due to overutilization and habitat destruction (TESHA 1991; AKETCH 1992; MARSHALL 1998). There is therefore a need to initiate conservation measures if the full medicinal potential of this plant is to be realised. According to MURCH *et al.* (2003), *in vitro* propagation of medicinal plants is a successful strategy that addresses the problems associated with supply and variability in product quality. This is achieved through enhancement of rapid mass production of true-to-type clones (WOCHOK 1981). The aim of this part of the study was to develop a viable micropropagation protocol for *Maytenus senegalensis* as a conservation option.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Seeds of *Maytenus senegalensis* were collected from a natural population in Machakos District, Eastern Province, Kenya in March 2000. The arils covering the seed (Plate 4.1 A) were manually removed by hand prior to mechanical scarification of the seed coat using sand paper. The scarified seeds were decontaminated by dipping in 70% (v/v) ethanol for I min and then agitating in 0.1% mercuric chloride supplemented with a few drops of Tween 20 for 12 min. The seeds were then thoroughly rinsed by agitating in three changes of sterile distilled water, each lasting at least 5 min. The decontaminated seeds were aseptically placed on hormone-free, ¼ strength modified MS medium (MURASHIGE and SKOOG 1962) solidified with 0.3% (w/v) gellan gum (Gelrite®). The

pH of the medium was adjusted to 5.8 using 1 N KOH and 1 N HCl before autoclaving for 20 min at 121 °C and a pressure of 103 kPa.

The seeds were maintained in the dark at 25±2 °C until germination occurred. Once germinated, the seedlings were removed and placed in a growth room with a light regime of 16/8 h light/dark photoperiod (27 µmol $m^{-2}s^{-1}$) provided by a mixture of cool white fluorescent tubes and incandescent lights and maintained at 25±2 °C in order to grow. The *in vitro* germinated seedlings were used as the source of explants for the subsequent experiments.

4.2.2 Explant selection, media and supplements

Four types of explants viz. nodal sections, leaves, petioles and roots were obtained from 6-week-old *in vitro* germinated seedlings and aseptically placed onto a shoot multiplication media containing hormones. The nutrient medium consisted of modified MS medium (MURASHIGE and SKOOG 1962) supplemented with 3% sucrose, 100 mgl^{-1} myo-inositol and 2 types of cytokinins, 6-benzylamino-purine (BA) and kinetin at 0, 0.5, 1.5 and 3.0 mgl^{-1}. The pH of the medium was adjusted to 5.8 and solidified with 0.3% Gelrite prior to autoclaving. The control experiment consisted of MS medium with no hormone supplementation. Explants were incubated under 16/8 h (light/dark) photoperiod at 25±2 °C. Eight replicates were used per treatment for each type of explant in a completely randomized design.

The experiment was carried out for a period of 12 weeks. However, the explants were placed on fresh medium every 4 weeks. Initially, the explants were placed on 10 ml nutrient medium and transferred onto 25 ml nutrient medium after 8 weeks. The responses of the explants to the respective plant growth regulators (PGRs) were expressed as the number of shoots (≥5 mm long) produced per explant at the end of the 12^{th} week. The lengths of the shoots (mm) were recorded. The experiment was conducted twice and the results averaged.

From these experiments, the best explant for shoot production was identified which in this case was the nodal explants. Nodal explants from 6-week-old *in vitro* germinated seedlings were placed onto a grid with concentrations of 0, 0.1, 0.5 and 1 mgl^{-1} BA/IAA and BA/IBA in a four by four-factorial combination. Ten replicates were used per treatment. The cultural conditions were the same as in the previous experiment.

After a period of 12 weeks, with monthly subcultures to freshly prepared nutrient medium as in the previous experiment, the response of the explants was expressed as the number of shoots (≥5 mm long) produced per explant. The lengths of the shoots (mm) were also measured. The experiment was conducted twice and the results averaged.

The optimal hormone combination and concentration for multiple shoot initiation and proliferation was determined. Once these criteria were fulfilled, multiple shoot multiplication was initiated using whole *in vitro* axillary shoots as explants. The shoots were serially subcultured every 3-4 weeks for a period of 16 weeks before rooting experiments were initiated. Shoot tip necrosis was a major problem but it was overcome by prompt subculture every 3–4 weeks and substitution of Gelrite with agar (0.8%) as a gelling agent in the shoot multiplication phase.

4.2.3 *In vitro* rooting

Since no rooting occurred in the shoot initiation and proliferation trials, experiments were conducted to induce rooting in 2-3 cm long, 8-week-old *in vitro* shoots. A series of experiments using MS medium supplemented with low concentrations (0, 0.5, 1.5 and 3.0 mgl^{-1}) of IAA, IBA and NAA were conducted. No rooting response was observed and hence the results are not presented. Another experiment was set up whereby isolated shoots were placed on filter paper bridges (Whatman No. 3) in ½ MS liquid medium containing 0 or 25 mgl^{-1} of IAA, IBA or NAA for 96 h in the dark at 28±2 °C for root induction. The shoots were then transferred to a hormone free ½ MS agar gelled (0.8%) medium at 28±2 °C in a dark/light regime of 12/12 h at 28±2 °C for root growth and development. Light (27 µmol $m^{-2}s^{-1}$) was provided by a mixture of cool, white fluorescent

tubes and incandescent lights. The number of roots produced per shoot was recorded 14 days after placement on the hormone-free, root growth and development medium. Twenty replicates were used for each treatment. The experiment was conducted twice and the results averaged. From these experiments, the best auxin was identified and used in subsequent experiments.

To optimize the concentration of the best auxin and the period of pulse treatment required, another experiment was set up in which three different concentrations of IBA (5, 12.5, 25 mgl^{-1}) in ½ MS liquid media were used to pulse-treat 8-week-old shoots for 24, 48, 72, 96 and 120 h in the dark at 28±2 °C. The shoots were then transferred to a hormone free ½ MS agar gelled (0.8%) medium at 28±2 °C in a dark/light regime of 12/12 h. The number of roots produced per shoot was recorded 14 days after the date of transfer to the root growth medium. Ten replicates were used for each treatment. The experiment was conducted twice and the results averaged. The treatment that gave the highest number of roots per plantlet was used to root plantlets for acclimatization purposes.

4.2.4 Acclimatization

Agar was carefully washed off the rooted plantlets to minimize pathogen attack (GEORGE 1996; COLLINS and EDWARDS 1998). The plantlets were planted on three types of potting mixtures, which included:

A. 1 sand: 1 peat: 1 vermiculite (v/v/v);
B. 1 potting soil: 1 sand: 1 perlite (v/v/v); and
C: 3 sand: 1 vermiculite (v/v).

The plantlets were placed in a mist house for a period of four weeks. They were then transferred to a green house (25±2 °C), where they were watered twice a day for the first two weeks and then once a day afterwards. The percentage survival was determined after two months. Fifty plantlets were planted for each type of potting mixture.

4.2.5 Statistical analysis

Mean values of the various treatments were compared by analysis of variance (ANOVA) using Minitab Release 12.1. Significance was accepted at p=0.05. Where significant differences were found, the means were separated using Tukey's family error test. In trials where the effect of more than one factor was being investigated, an analysis of variance (ANOVA) was done using sum of square for tests in a general linear model.

4.3 RESULTS AND DISCUSSION

4.3.1 Shoot initiation and multiplication

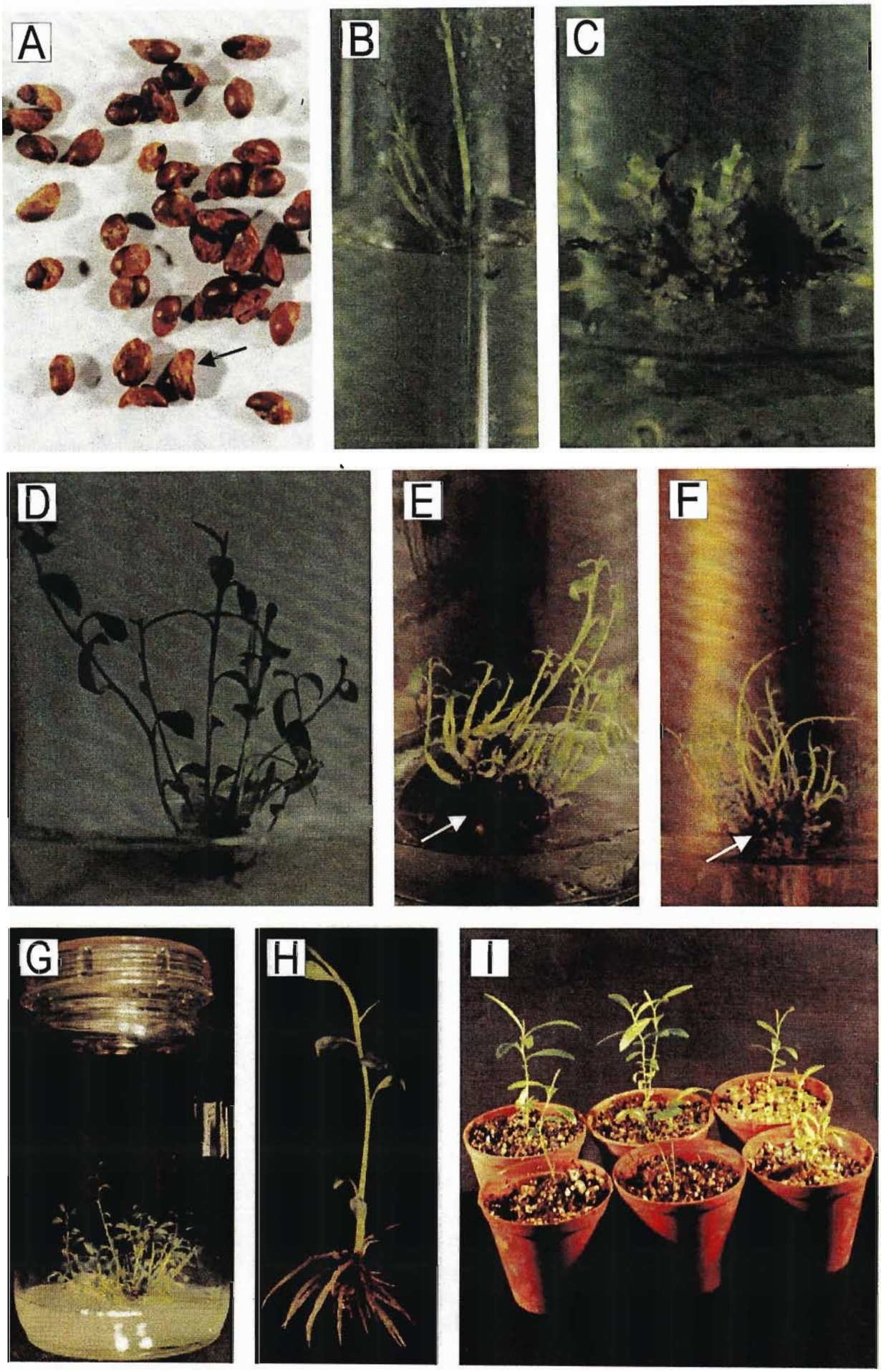
Seeds of *M. senegalensis* were sterilized successfully. Over 70% of the seeds germinated *in vitro* providing a source of sterile explants.

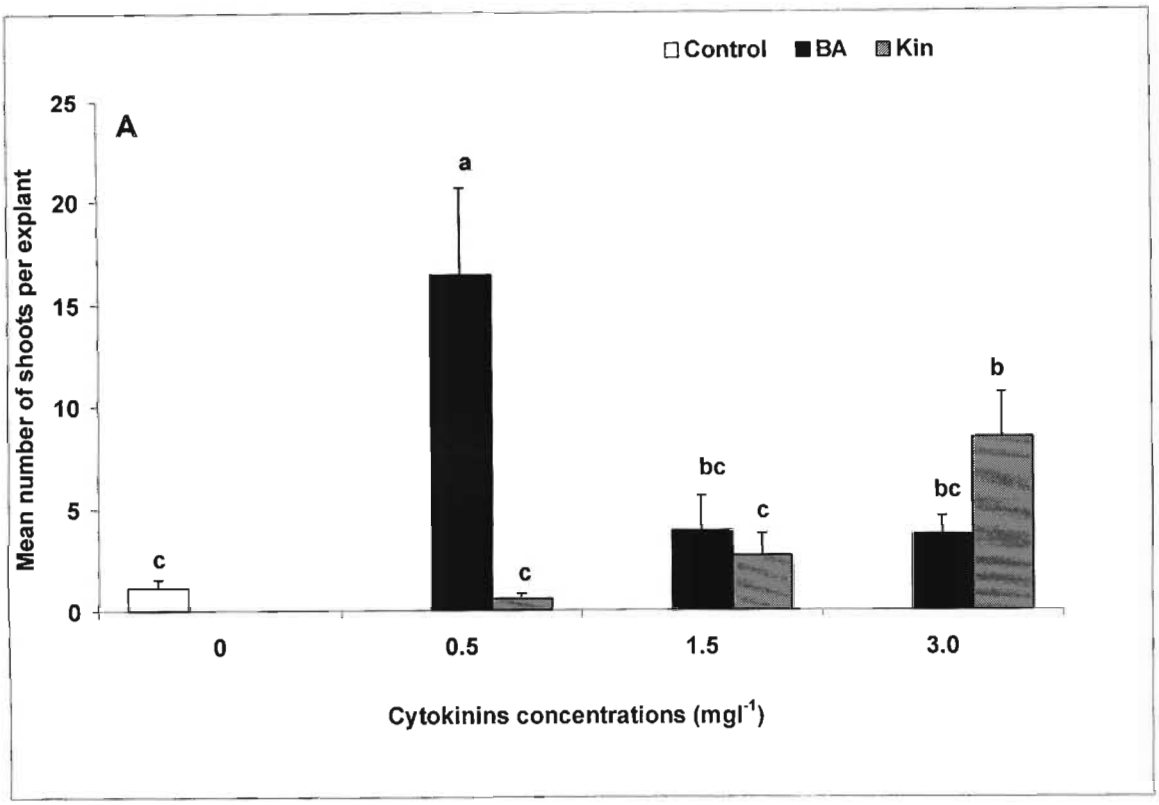
4.3.1.1 Explant selection

Nodal explants were the only ones that produced multiple shoots. All the explants obtained from the leaf sections and the petioles turned brown and died. In the medium containing 0.5 mgl^{-1} kinetin, the initial root explants either extended or produced new roots. However, root explants placed on other treatments turned black with no observable morphogenic responses.

Multiple shoots were initiated directly from the nodal explants (Plate 4.1B). The type and concentration of cytokinins influenced the average number of shoots produced per explant (Fig. 4.1A) as well as the mean length of the shoots (Fig. 4.1B). Explants placed on medium containing 1.5 and 3.0 mgl^{-1} BA produced clusters of stunted shoots that averaged about 2 mm in length (Plate 4.1C). Dwarf shoots were formed with high concentrations of BA in other members of the family Celastraceae such as *Catha edulis* (ELHAG 1991), *Maytenus emarginata* (RATHORE *et al.* 1992) and *M. ilicifolia*

Plate 4.1 Stages of *in vitro* propagation of *M. senegalensis*. **A:** Seeds of *M. senegalensis* (arrow showing the aril), **B**: Multiple shoot production by nodal explants on a medium containing 0.5 mgl^{-1} BA after 4 weeks, **C**: Clusters of stunted shoots on a medium containing 3 mgl^{-1} BA after 12 weeks, **D**: Multiple shoot production on a medium containing 3.0 mgl^{-1} kinetin after 12 weeks, **E**: Black nodular swellings at the base of the shoots on a medium containing 1 mgl^{-1}BA/1 mgl^{-1} IAA after 12 weeks, **F**: Production of stunted shoots on a medium containing 1 mgl^{-1} BA after 12 weeks, **G**: Shoot multiplication on a medium containing 0.5 mgl^{-1} BA after 8 weeks, **H**: Rooted *M. senegalensis in vitro* shoot, **I**: Acclimatized 2 month old *M. senegalensis* plantlets.


A
B
C
D
E
F
G
H
I



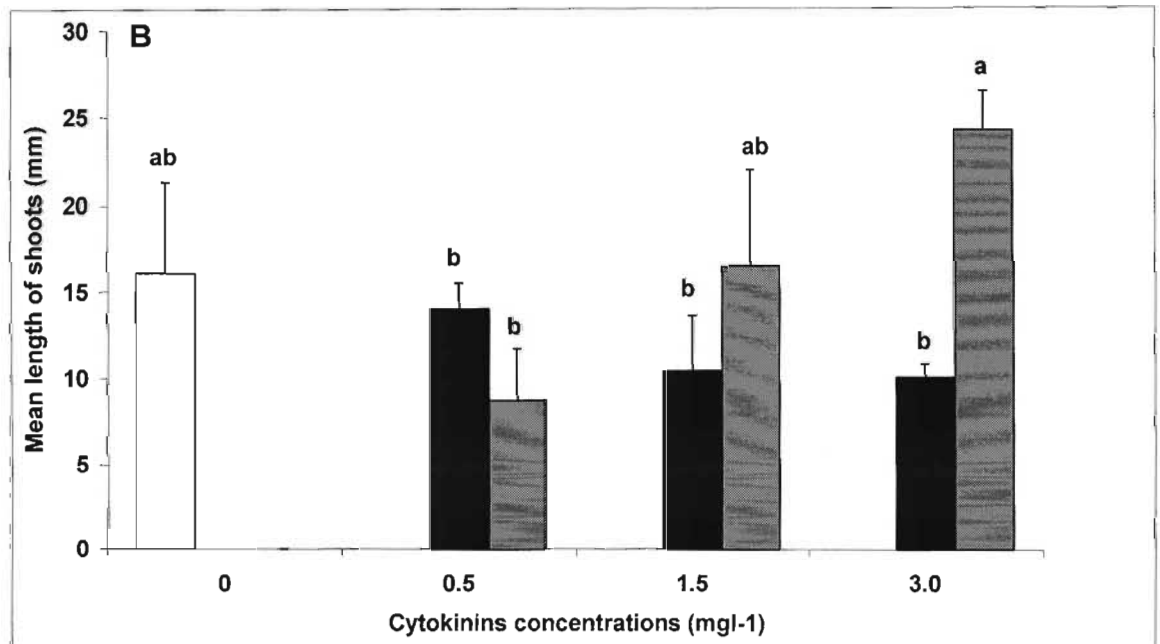


Fig 4.1 The average number (A) and the mean length (B) of shoots initiated per *M. senegalensis* nodal explant after 12 weeks on a medium containing cytokinins. (Bars followed by the same letter(s) are not siginificantly different at p=0.05).

(PEREIRA *et al.* 1995). Very few stunted shoots were observed in the treatments containing kinetin (results not shown).

The highest number of shoots produced per explant was on medium containing 0.5 mgl^{-1} BA. Generally, an increase in BA concentration led to a significant decrease ($p<0.001$) in the number of shoots (≥5 mm long) produced per explant. On the other hand, an increase in kinetin concentration resulted in a significant increase ($p<0.001$) in the number of shoots produced per explant (Fig 4.1 A). This observation implied that the optimum kinetin concentration on the average number of shoots produced per nodal explant had not been reached at a concentration of 3.0 mgl^{-1}. The least number of shoots produced per explant was in the treatment containing 0.5 mgl^{-1} kinetin but there were no significant differences in the number of shoots produced per explant in this treatment when compared with the control (Fig 4.1 A).

Although nodal explants placed on medium containing BA had a tendency to produce stunted shoots (Plate 4.1C), BA was superior to kinetin in terms of the overall number of shoots produced per explant (Fig 4.1 A). Kinetin was found to be less effective than BA in shoot initiation and proliferation in some woody plants such as *Bougainvillea glabra* "Magnifica" (SHARMA *et al.* 1981) and *Catha edulis* (ELHAG 1991).

The longest shoots were produced in the treatment containing kinetin (3.0 mgl^{-1}) and they were significantly longer ($p=0.04$) compared to shoots produced in some treatments (Fig 4.1B). The length of shoots increased with an increase in the concentration of kinetin. A reverse response was observed in treatments containing BA whereby the length of shoots decreased though not significantly with an increase in concentration (Fig. 4.1B).

Hyperhydricity of shoots was observed in treatments containing 3.0 mgl^{-1} BA. However, this undesirable development was overcome by placing the shoots in a medium containing 15 gl^{-1}activated charcoal (AC). A high level of BA in the medium was found to be the major factor influencing hyperhydricty in *Camelia sinensis*, *Gerbera jamesonii*, *Malus domestica* and a *Poplar* hybrid (KATAEVA *et al.* 1991).

Activated charcoal is known to adsorb high concentration of plant growth regulators (PGRs) such as BA (PAN and VAN STADEN 1998).

It is apparent that the presence of exogenous cytokinins is important for shoot induction and growth in *M. senegalensis* since very few shoots were produced in the absence of cytokinins (Fig 4.1A). The importance of cytokinins has also been demonstrated in *in vitro* shoot production of other members of the Celastraceae such as *Catha edulis* (ELHAG 1991), *Maytenus emarginata* (RATHORE *et al.* 1992), *M. Ilicifolia* (PEREIRA *et al.* 1995) and *M. canariensis* (MEDEROS-MOLINA 2002). Cytokinins promote axillary shoot formation by inhibiting apical dominance (PIERIK 1987).

BA at lower concentration (0.1-1.0 mgl^{-1}) was chosen for the subsequent experiments that aimed at investigating the synergistic effects of BA and low concentrations of auxins (IAA and IBA) on shoot initiation and growth.

4.3.1.2 Effects of BA and auxins on shoot initiation and growth

In the trials that aimed at investigating the effects of BA/IAA and BA/IBA, BA was the only plant growth regulator that had a significant main effect on both the average number of shoots initiated per nodal explant (Table 4.1 and Table 4.3) and the mean length of shoots (Table 4.2 and Table 4.4). There were no significant interactions between BA and IAA (Table 4.1 and Table 4.2) or BA and IBA (Table 4.3 and Table 4.4) on the two parameters recorded viz. average number of shoots (≥5 mm long) produced per explant and the mean length of shoots.

Shoot initiation and growth in the two sets of experiments had a similar pattern (Table 4.5). The number of shoots produced per explant was higher in the medium containing 0.5 and 1.0 mgl^{-1} BA regardless of the presence or absence of either IAA or IBA. These results imply that the average number of shoots produced per nodal explant was not influenced by the addition of either IAA or IBA at any of the tested concentrations. Similar observations were made for *M. ilicifolia* (PEREIRA *et al.*1995).

Table 4.1 Analysis of variance showing the effect of various concentrations of BA and IAA on the average number of shoots (≥5 mm long) produced per *Maytenus senegalensis* nodal explant after 12 weeks.

Source of variation	df	Sum of squares	F value	p value*
BA	3	10775	136.75	<0.001
IAA	3	115.9	1.47	0.287
BA x IAA	9	236.4	0.17	0.997
Error	144	22884.9		

*Significance at p=0.05

Table 4.2 Analysis of variance showing the effect of various concentrations of BA and IAA on the mean length of shoots produced per *Maytenus senegalensis* nodal explant after 12 weeks.

Source of variation	df	Sum of squares	F value	*p value
BA	3	3829.5	10.24	0.003
IAA	3	839.1	2.24	0.153
BA X IAA	9	1122.4	0.83	0.587
Error	144	21576.6		

* Significance at p=0.05

Table 4.3 Analysis of variance showing the effect of various concentrations of BA and IBA on the average number of shoots (≥5 mm long) produced per *Maytenus senegalensis* nodal explant after 12 weeks.

Source of variation	df	Sum of squares	F value	*p value
BA	3	8010.5	130.91	<0.001
IBA	3	149.9	2.43	0.132
BA x IBA	9	183.6	0.18	0.996
Error	144	16406.8		

*Significance at p=0.05

Table 4.4 Analysis of variance showing the effect of various concentrations of BA and IBA on the mean length of shoots produced per *Maytenus senegalensis* nodal explant after 12 weeks.

Source of variation	df	Sum of squares	F value	*p value
BA	3	4231.2	11.85	0.002
IBA	3	439.9	1.23	0.354
BA x IBA	9	1071.0	0.91	0.520
Error	144	18872.0		

*Significance at p=0.05

Table 4.5 The average number (±SE) and the mean length (±SE) of shoots initiated per *Maytenus senegalensis* nodal explant after 12 weeks on a medium containing various concentrations of BA/IAA or BA/IBA. (Mean values in the same column followed by the same letter(s) do not differ significantly at p=0.05).

Hormone concentrations (mgl^{-1})		BA:IAA		BA:IBA	
		Morphogenic response			
BA	IAA/IBA	Average No. of shoots	Mean length of shoots (mm)	Average No. of shoots	Mean length of shoots (mm)
0	0	1.3 ± 0.3d	15.8 ± 2.7abcd	2.0 ± 0.5e	15.5 ± 4.1bcde
0	0.1	1.0 ± 0.2d	25.7 ± 6.1abcd	1.0 ± 0.3e	22.8 ± 3.9de
0	0.5	1.7 ± 0.4d	27.7± 6.6abc	1.9 ± 0.5e	23.4 ± 4.8abc
0	1.0	1.2 ± 0.3d	26.9 ± 5.2abc	1.1 ± 0.3e	25.5 ± 5.9ab
0.1	0	3.7 ± 1.1cd	21.3 ± 5.3abcde	6.7 ± 1.8bcde	18.3 ± 3.6abcde
0.1	0.1	6.5 ±1.1bcd	29.2 ± 2.4ab	6.2 ± 1.1cde	28.4 ± 3.7a
0.1	0.5	5.0± 1.2cd	31.9 ± 4.9a	5.1 ± 0.9de	26.7 ± 4.6
0.1	1.0	4.0 ± 0.8cd	24.1 ± 4.7abcde	4.6 ± 1.1de	20.6 ± 3.9abcd
0.5	0	18.9 ± 3.4ab	17.1 ± 2.6bcde	20.7 ± 6.2a	15.5 ± 2.7bcde
0.5	0.1	18.1 ± 1.0ab	16.0 ±2.0cde	16.2 ± 4.3abc	11.6 ± 2.8de
0.5	0.5	22.9 ± 3.0a	14.7 ± 2.8de	18.5 ± 3.8a	16.4 ± 2.5bcde
0.5	1.0	15.3 ± 2.4abc	18.3 ± 3.1bcde	17.9 ± 4.4a	12.6 ± 3.4cde
1.0	0	19.2 ± 3.1a	12.1 ± 2.1e	17.6 ± 3.9a	11.6 ± 2.5de
1.0	0.1	19.5 ± 2.3a	17.3 ± 1.5bcde	18.0 ± 4.1a	8.8 ± 2.2e
1.0	0.5	20.2 ± 4.0a	12.7 ± 2.2e	17.3 ± 4.8ab	13.2 ± 2.4cde
1.0	1.0	20.0 ± 1.5a	18.0 ± 1.5bcde	12.6 ± 5.3abcd	11.5 ± 2.6de

Low levels (0.1 mgl^{-1}) or the absence of BA generally promoted the length of shoots (Table 4.5). The longest shoots (31.9 mm) were produced in the medium containing BA:IAA (0.1 mgl^{-1}:0.5 mgl^{-1}).

Nodal explants placed on medium containing 0.5 and 1.0 mgl^{-1} IAA or IBA in the presence of 0.5 and I.0 mgl^{-1} BA produced black, nodular swellings at the base (Plate 4.1E). Stunted shoots were produced in the medium containing 1.0 mgl^{-1} BA (Plate 4.1 F) in the presence or absence of either IAA or IBA (results not shown). Shoot tip necrosis was initially a major problem. However, it was overcome by placing the shoots on a medium solidified with 0.8% agar instead of 0.3% Gelrite at the shoot multiplication stage.

The results indicated that 0.5 or 1.0 mgl^{-1} BA in the absence or presence of low (0.1 or 0.5 mgl^{-1}) IAA or IBA was optimal for shoot induction and growth in terms of the high number of shoots produced per explant. However, incorporation of 0.5 mgl^{-1} BA alone in the shoot multiplication medium was eventually chosen. This concentration was preferred over 1.0 mgl^{-1} BA as no undesirable responses were observed at this concentration. It is also a good strategy in terms of reduction of cost of production. Auxins were excluded, as they did not confer any extra beneficial physiological responses and their exclusion would further reduce the cost of production. High cost of production has been cited as one of the limitations of application of plant tissue culture (GOVIL and GUPTA 1997).

Shoots were multiplied (Fig 4.1G) by using whole *in vitro* shoots (2-3 cm long) as explants. The explants were obtained from the previous trial containing 0.5 mgl^{-1} BA alone. The shoots were bulked up by serial subculture every 3-4 weeks for a period of four months before *in vitro* rooting trials commenced.

4.3.2 *In vitro* rooting

Rooting only occurred on shoots that were placed on auxins (Fig 4.2). The number of roots produced per shoot was significantly higher ($p<0.001$) in the treatment containing IBA compared to IAA or NAA. There were no significant differences on the average number of roots produced per shoot in the treatments containing IAA and NAA (Fig 4.2). IBA was hence more effective in promoting *in vitro* rooting compared to IAA and NAA. IBA was found to be more effective in rooting of some Australian woody plant species (WILLIAMS *et al.* 1985).

Emergence of root initials did not occur during the root induction phase and only started 4-5 days after transfer to the hormone-free, root growth medium. Auxins are involved in adventitious root formation (WYNNE and McDONALD 2002) in many woody plant species (PIERIK 1987). However, continued presence of auxins may inhibit emergence of root initials and thus transfer of shoots to an auxin-free medium is preferred (GEORGE 1996).

Although the concentration of the auxins used was relatively high (25 mgl^{-1}), callus formation was not observed. Contact of tissues with a high concentration of auxins may lead to formation of callus (GEORGE 1996: COLLINS and EDWARDS 1998). There was erratic elongation of the upper internodes in some shoots.

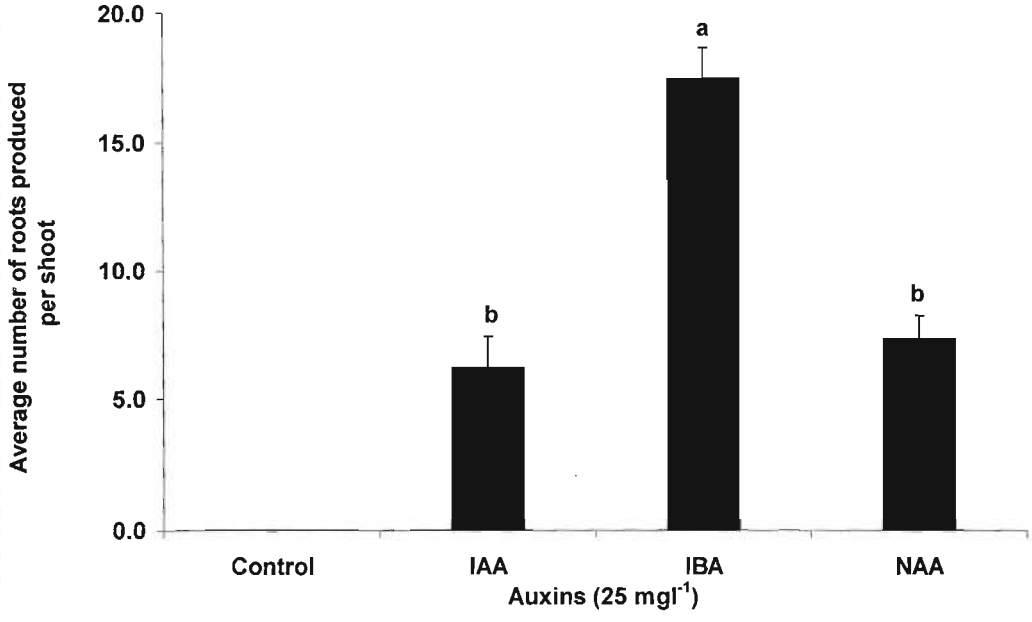


Fig. 4.2 Effect of auxins on the average number of roots produced per *M. senegalensis* shoot, 14 days after transfer to a hormone-free medium. (Bars with different letters are significantly different at p=0.05).

In an attempt to optimize the IBA concentration and the period of pulse treatment in the root induction phase, the results showed that each of the two factors had a significant effect on their own and there was a significant interaction between them (Table 4.6).

Table 4.6 Analysis of variance showing the effect of different concentrations of IBA and different periods of pulse treatment on the average number of roots produced per *Maytenus senegalensis* shoot, 14 days after transfer to a hormone-free medium.

Source of variation	df	Sum of squares	F value	*p value
IBA	2	1860.0	10.3	0.003
Time	4	2426.0	15.8	0.002
IBA x Time	8	472.1	2.6	0.010
Error	135	3019.6		

*Significance at p=0.05

At a concentration of 5 mgl^{-1} IBA, fewer numbers of roots were produced per shoot regardless of the period of pulse treatment (Table 4.7). The average number of roots produced per shoot increased with an increase in the period of pulse treatment. At this concentration, the average number of roots produced by shoots pulse-treated for 120 h was significantly higher ($p<0.001$) compared to those pulse-treated for 24 and 48 h respectively (Table 4.7).

In the treatments receiving 12.5 mgl^{-1} IBA, the average number of roots produced by shoots pulse-treated for 96 and 120 h was significantly higher compared to those pulse-treated for 24, 48 and 72 h and all those in the medium containing 5 mgl^{-1} IBA. At 25 mgl^{-1} IBA, a high number of roots was produced by shoots pulse–treated for 72 and 96 h. The overall highest number of roots produced per shoot, was in the shoots pulse-treated in 25 mgl^{-1} IBA for 120 h and was significantly higher than all the other treatments (Table 4.7). This treatment was hence chosen to root plantlets that were acclimatized (Plate 4.1H).

Table 4.7 The influence of various concentrations of IBA and different periods of pulse treatment on the average number of roots produced per *Maytenus senegalensis* shoot, 14 days after transfer to a hormone-free medium. (Mean values (±SE) in the same column followed by different letter(s) are significantly different at p=0.05).

IBA concentration (mgl^{-1})	Period of pulse treatment (h)	Average number (±SE) of roots per shoot
5	24	0.6 ± 0.3e
	48	0.8 ± 0.5e
	72	2.8 ± 0.8cde
	96	3.3 ± 0.9cde
	120	6.6 ± 1.2c
12.5	24	1.6 ± 0.7de
	48	2.1 ± 0.8cde
	72	5.1 ± 1.5cde
	96	13.0 ± 1.8b
	120	12.5 ± 1.7b
25	24	5.6 ± 1.3cd
	48	6.5 ± 1.7c
	72	11.5 ± 2.2b
	96	13.1 ± 2.5b
	120	20.5 ± 1.5a

In plant species that require the addition of exogenous auxins to root, the numbers of roots produced per shoot usually increases proportionally with an increase in auxin concentration, until the optimum level is reached (GEORGE 1996). It is hence interesting that the optimum IBA concentration would be higher than 25 mgl^{-1} when rooting *M. senegalensis in vitro* shoots. RATHORE *et al.* (1992) reported the requirement of such high concentrations of IBA in rooting *M. emarginata in vitro* shoots.

4.3.3 Acclimatization

M. senegalensis plantlets were successfully acclimatized (Plate 4.1l) in the three potting mixtures investigated. The percentage survival after a period of 8 weeks was 86% in potting mixture B (1 potting soil: 1 sand: 1 perlite v/v/v) compared to 76% for potting mixture C (3 sand: vermiculite v/v) and 73% for potting mixture A (1 peat: l vermiculite: 1 sand v/v/v).

4.4 CONCLUSIONS

An efficient micropropagation protocol for *M. senegalensis* was established. Using this protocol, it is possible to generate over 5,000 plantlets from a single node over a period of 30 weeks. Such generated plantlets can be used to replenish the declining populations in the wild and/or can be cultivated by Traditional Medical Practitioners (TMPs) in their gardens thus reducing pressure off wild stocks.

The requirements for the presence of cytokinins in shoot induction and growth and auxins in adventitious root formation in *Maytenus senegalensis* were demonstrated, phenomena observed in other *Maytenus* spp such as *Maytenus canariensis* (MENDEROS-MOLINA 2002), *M. emarginata* (RATHORE *et al.* 1992) and *M. ilicifolia* (PEREIRA *et al.* 1995).

CHAPTER 5

THE INFLUENCE OF SOME FACTORS ON ADVENTITIOUS ROOT FORMATION IN *MAYTENUS SENEGALENSIS*

5.1 INTRODUCTION

Rooting of *in vitro* grown shoots largely contributes to the efficiency of a micropropagation protocol (MONCOUSIN 1991a; ROGERS and SMITH 1992; COLLINS and EDWARDS 1998). This is mainly because a good rooting system permits successful transfer of plantlets from the *in vitro* to the greenhouse and field conditions (BADZIAN *et al.* 1991; PANAIA *et al.* 2000). However, adventitious root formation remains a major obstacle in micropropagation (DE KLERK 2002), particularly of woody plant species (DURZAN 1988; JONES 1991). Huge losses can be incurred due to non-rooted shoots, poor rooting per individual and subsequent poor quality of the developed root system.

Adventitious root formation is an organized developmental process involving discreet biochemical, physiological and histological events (DAVIES and HARTMANN 1988). The rooting phenomenon occurs in a series of stages (DAVIES and HARTMANN 1988; MONCOUSIN 1991b; McDONALD and WYNNE 2003). The first stage is the root induction stage. It involves the determination of the root formation capacity and is marked by cytological modifications. The second stage is the root initiation stage, which is marked by the appearance of cellular divisions ending with the constitution of a primary mass of meristematic cells (morphogenetical rooting zones). Organization of the morphogenetical rooting zones occurs in the third stage. The fourth stage involves the growth of the root meristem (root elongation) and it is characterized by the development of the root primordia into roots. Each of these stages has varying sensitivities to any external environment (MONCOUSIN 1991b). Consequently, treatments, which promote events in one stage, may not be optimal to those that precede or follow (GEORGE 1996).

Interactions of many endogenous and exogenous factors influence the ability of axillary shoots to root (GEORGE 1996). PIERIK (1987) listed a number of these factors, some of which include: auxins, temperature, light, agar, sugar, mineral nutrients and activated charcoal.

Attempts to root *Maytenus senegalensis* in *vitro* shoots were met with difficulties (Section 4.2.3, Chapter 4). Rooting only occurred after pulse-treating the shoots with high concentrations of auxins. *M. senegalensis* can therefore be classified as a difficult-to-root plant species. *In vitro* propagation is known to offer an excellent facility to study the factors that determine the rooting ability of shoots (WYNNE and McDONALD 2002).

The aim of this part of the study was to determine the effects of some factors on adventitious root formation in *M. senegalensis in vitro* grown shoots. The factors investigated were:

1. Various IBA concentrations and different temperatures;
2. Light (presence or absence) and media type (agar gelled or liquid);
3. Different sucrose concentrations;
4. Different MS media strengths; and
5. Activated charcoal.

5.2 MATERIALS AND METHODS

5.2.1 Source of shoots for rooting experiments

The shoots that were used in the rooting experiments were initiated from nodal explants obtained from 6-week-old *in vitro* germinated seedlings (Section 4.3.1.2, Chapter 4). Shoot multiplication was done using whole *in vitro* shoots as explants. The shoot multiplication medium comprised of MS salts (MURASHIGE and SKOOG 1962) supplemented with BA (0.5 mgl^{-1}), sucrose (30 gl^{-1}) and myo-inositol (100 mgl^{-1}). The pH of the medium was adjusted to 5.8 using 1 N KOH and 1 N HCl before the addition of 0.8% agar (Agar-Agar Powder CP, Associated Chemical Enterprises C.C. SA) and then autoclaved at 121 °C for 20 min. The cultures were placed in a growth room with a light regime of 16/8 h (light/dark) (27 µmol m^{-2} s^{-1}) provided by a mixture of cool white fluorescent tubes and incandescent lights and maintained at 25±2 °C. The proliferating shoots were placed onto fresh medium every 3-4 weeks.

5.2.2 Rooting

Rooting was done in two stages viz. root-induction and root-growth. In the root-induction stage, single 8-week-old shoots (2-3 cm long) were excised and pulse-treated with IBA (25 mgl^{-1}) in a ½ strength MS liquid medium in the dark at 28 °C (unless otherwise stated) for 120 h. In the root-growth stage, the pulse-treated shoots were transferred to a hormone-free, ½ strength MS medium solidified with 0.8% agar (unless otherwise stated). The cultured shoots were placed in a growth chamber maintained at 28 °C (unless otherwise stated) and a 12/12 h (light/dark) photoperiod (27 µmol m^{-2} s^{-1}) provided by a mixture of cool white fluorescent tubes and incandescent lights. Whenever a liquid medium was used, the shoots were placed on filter paper bridges (Whatman No. 3).

Both the root-induction and root-growth media were supplemented with sucrose (30 gl^{-1}) with the exception of the experiment in which the effects of different sucrose concentrations were being investigated. Myo-instol (100 mgl^{-1}) was also added. The pH of the medium was adjusted to 5.8 before autoclaving. Each shoot was placed onto 10 ml medium and the number of roots produced per shoot was recorded 14 days after transfer to the root-growth (hormone-free) medium.

5.2.2.1 Effect of IBA concentrations and temperature

The effects of different IBA concentrations (0, 5 12.5 and 25 mgl^{-1}) and different temperatures (15, 23, 28 and 35 °C) on adventitious root formation were evaluated. Rooting was totally inhibited in the absence of IBA and in all the shoots that were placed at 15 °C. These results are hence not shown. At the end of this experiment, maximum rooting was found when the shoots were pulse-treated with 25 mgl^{-1} IBA and placed at 28 °C. Consequently, shoots were pulse–treated with 25 mgl^{-1} IBA and placed at 28 °C in subsequent experiments.

5.2.2.2 Effect of different sucrose concentrations

To determine the optimum sucrose concentration, the effect of different sucrose concentrations (10, 20, 30 40, 60 and 100 gl^{-1}) on adventitious root formation was tested. The control treatment contained no sucrose.

5.2.2.3 Effect of light and media type

The effect of light (presence or absence) and 0.8% agar (presence or absence) on adventitious root formation was evaluated. The shoots were placed in a liquid or agar-gelled medium and either kept under light or dark conditions. Dark-treated shoots were pre-treated with 120 h of total darkness and then transferred to 12/12 h photoperiod. Light-treated shoots were subjected to 12/12 h photoperiod (27 µmol m^{-2} s^{-1}) in both the root-induction and root-growth stage. The control treatment for this experiment

comprised of shoots pulse-treated in a liquid medium in the dark, and then transferred to an agar-gelled medium and placed at 12/12 h photoperiod.

5.2.2.4 Effect of media strength

The effect of four MS medium (MURASHIGE and SKOOG 1962) concentrations (1/4, 1/2, 3/4 and full strength) on adventitious root formation was determined.

5.2.2.5 Effect of activated charcoal

The effect of two concentrations (5 and 10 gl^{-1}) of activated charcoal (Sigma) on adventitious root formation was evaluated. Activated charcoal was only added to the hormone-free root-growth medium after the shoots had been pulse-treated with 25 mgl^{-1} IBA. The control treatment comprised of an agar-gelled medium without activated charcoal.

5.2.2.6 Statistical analysis

Ten replicates were used for each treatment. All the experiments were conducted at least twice and the results averaged. Mean values of the number of roots produced per shoot were compared by an analysis of variance (ANOVA) using Minitab Release 12.1. Significance was accepted at $p=0.05$. Where significant differences were found, the means were separated using Tukey's family error test. In trials where the effects of more than one factor were being investigated, an ANOVA was done using sum of squares in a general linear model.

5.3 RESULTS AND DISCUSSION

In all the treatments in which rooting occurred, there was no visible root development until 4-5 days after the shoots were transferred to the hormone-free root-growth medium.

5.3.1 Effect of IBA concentration and temperature

Rooting was induced over the entire range of IBA concentrations tested (Fig. 5.1). However, no rooting was observed in the control (Plate 5.1A) and all the treatments placed at 15 °C (results not shown). The different IBA concentrations and temperatures had significant effects on their own but there was no significant interaction between them (Table 5.1).

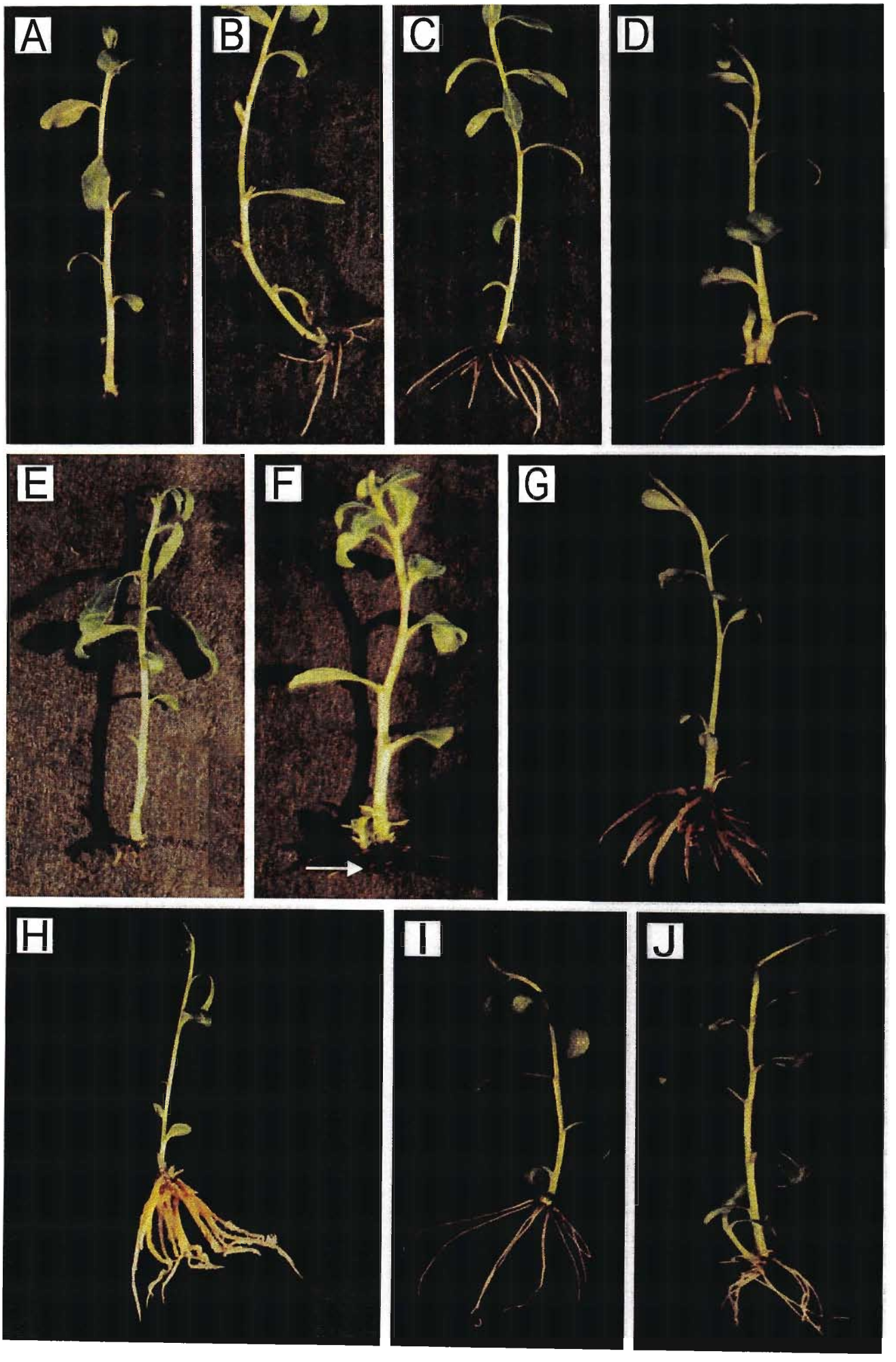
Table 5.1 ANOVA showing the effect of IBA concentrations and temperatures on the average number of roots produced per *Maytenus senegalensis* shoot, 14 days after transfer to a hormone-free medium.

Source of variation	df	Sum of squares	F value	*p
Temperature	2	545.1	6.7	0.05
IBA	2	1421.4	17.4	0.011
Temperature x IBA	4	163.5	1.4	0.257
Error	81	2442.6		

*Significance at p=0.05

The average number of roots produced per shoot increased significantly ($p<0.001$) with an increase in IBA concentration regardless of the incubation temperature (Fig. 5.1) and (Plates 5.1 B, C and D). These results confirm the significance of auxins on the rooting of *M. senegalensis in vitro* shoots because no rooting occurred in the absence of IBA at any of the temperature levels. It has been shown that some difficult-to-root woody plant species can be induced to root by the addition of exogenous auxins (WILLIAMS *et al.* 1985; SRIKANDARAJAH and MULLINS 1987; WYNNE and McDONALD 2002).

Plate 5.1 Adventitious root formation in *M. senegalensis in vitro* grown shoots, 14 days after transfer to a hormone free medium. **A**: An un-rooted shoot in the absence of IBA; **B**: A rooted shoot on a medium containing 5 mgl^{-1} IBA; **C**: A rooted shoot on a medium containing 12.5 mgl^{-1} IBA; **D**: A rooted shoot on a medium containing 25 mgl^{-1} IBA, **E**; Stunted, black-tipped roots formed in the absence of sucrose, **F**: Root formation on a medium containing 100 gl^{-1} sucrose (arrow showing a swollen shoot base), **G**: Root formation on an agar-gelled medium; **H**: Root formation on a shoot placed on a filter paper bridge and soaked in a liquid medium; **I**: Root formation in the presence of 5 gl^{-1} activated charcoal, **J**: Root formation in the presence of 10 gl^{-1} activated charcoal.


A
B
C
D
E
F
G
H
I
J

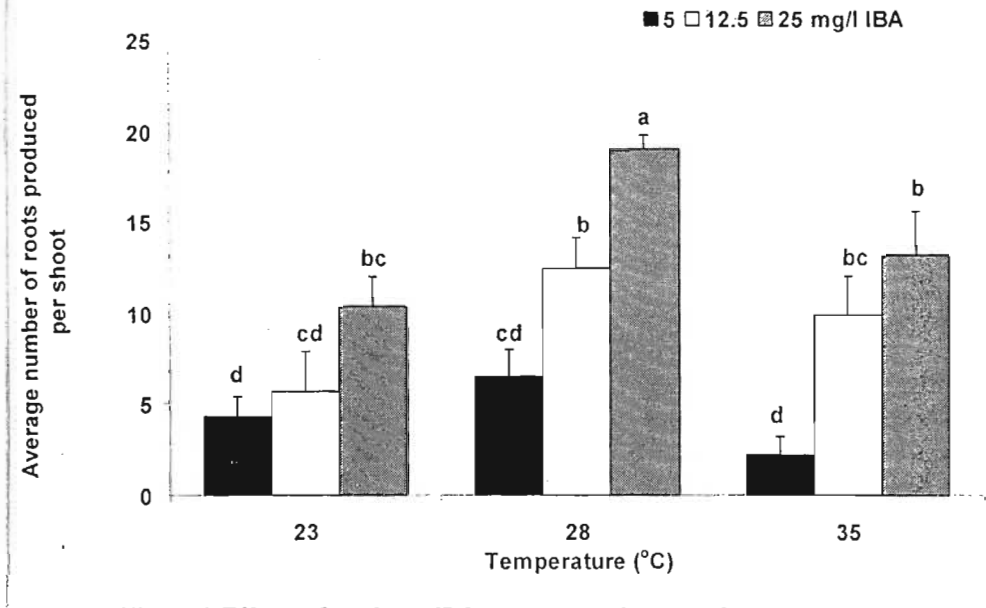


Fig. 5.1 Effect of various IBA concentrations and temperatures on the average number of roots produced per *M. senegalensis* shoot, 14 days after transfer to a hormone-free medium. (Bars with different letters are significantly different at p=0.05).

The average number of roots produced per shoot was higher at 28 °C compared to the shoots placed at 23 and 35 °C. The overall highest number of roots per shoot were produced in the shoots pulse-treated with 25 mgl^{-1} IBA and placed at 28 °C. Shoots placed at 23 °C generally produced fewer roots compared to those placed at higher temperatures (Fig. 5.1). The rate of cellular metabolism is increased at higher temperatures, a phenomenon that favours the initiation of the root primordium (MONCOUSIN 1991b).

High temperatures (35° C) affected the timing of root emergence and the shoot quality. At 23 and 28 °C, root emergence started 4-5 days after the shoots were transferred to the root-growth medium and no shoot senescence was observed. At 35 °C, roots emerged 2-3 days after transfer to the root-growth medium. Browning (senescence) of the shoots was observed at 35 °C. Temperatures above the optimal levels were found to influence rooting of some Rose cultivars by affecting the timing of root emergence (ALDERSON *et al.* 1988).

Adventitious root formation is a temperature-dependent process (GEORGE 1996). The results obtained in this study confirmed this observation. A higher number of roots were produced by shoots placed at 28 °C compared to the shoots placed at 23 and 35 °C. This implies that 28 °C is the optimal temperature for adventitious root formation in *M. senegalensis*. *In vitro* shoots of *M. emarginata* were found to produce the maximum number of roots at 28 °C as well (RATHORE *et al.* 1992).

5.3.2 Effect of different sucrose concentrations

The effect of different sucrose concentrations on the average number of roots produced by *M. senegalensis in vitro* grown shoots are shown on Fig. 5.2. Adventitious root formation was significantly influenced by different sucrose concentrations ($p<0.001$). A good rooting response was evident at a wide range of sucrose concentrations (10-60 gl^{-1}). No significant influence was found on the average number of roots produced per shoot at this concentration range.

In many plant species, sucrose concentrations in the range between 20-30 gl^{-1} are optimal for adventitious root formation (GEORGE 1996). However, some plants require abnormally high levels (90 gl^{-1}) while others respond to a wide range (15-60 gl^{-1}) of sucrose concentrations (GEORGE 1996).

The absence or the presence of a high sucrose concentration (100 gl^{-1}) significantly reduced the average number of roots produced per shoot ($p<0.001$). The shoots that were placed on the medium lacking sucrose produced few, stunted (<3 mm long) and black-tipped roots (Plate 5.1E). The shoots that were placed on a medium containing high sucrose concentration (100 gl^{-1}) produced a few healthy roots but the shoot bases were extensively swollen (Plate 5.1F). In a *Vitis* hybrid 'Remaily Seedless', sucrose concentrations above the optimal level were found to inhibit rooting (CHÉE and POOL 1988).

Adventitious root formation is an energy-requiring process (ZIMMERMAN 1983). A requirement for sugars has therefore been established in many plant species (PIERIK 1987). Sugars can also be supplied to the shoot through the process of photosynthesis. However, an exogenous supply is always essential because the conditions under which the in *vitro* shoots are grown are not optimal for photosynthesis (GEORGE 1996). During adventitious root formation, sugars can play nutritional and/or osmotic roles (CONNER and FALLOON 1993).

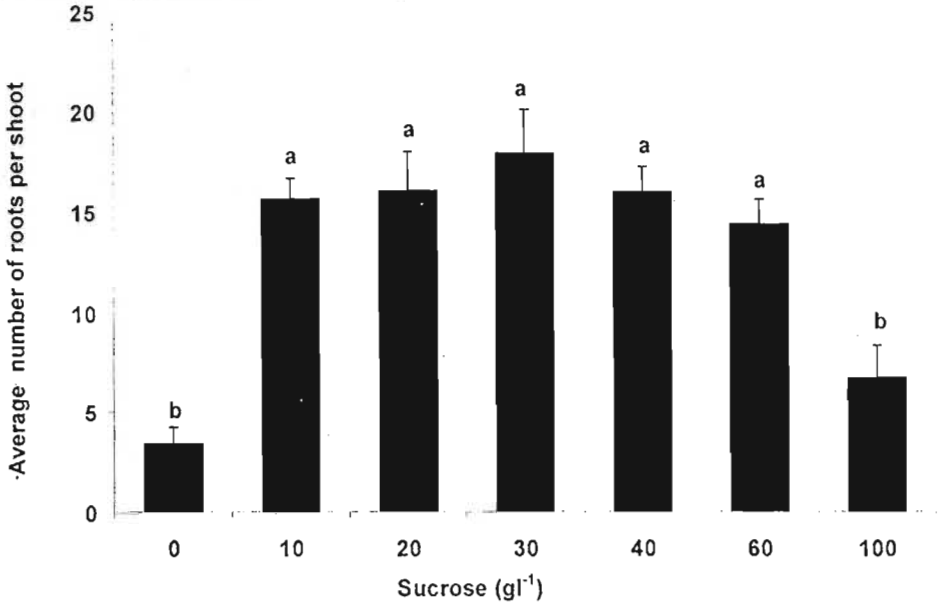


Fig. 5.2 Effect of different sucrose concentrations on the average number of roots produced per *M. senegalensis* shoot, 14 days after transfer to a hormone-free medium. (Bars bearing different letters are significantly different at p=0.05).

5.3.3 Effect of media type and light

The effect of media type (agar-gelled or liquid) and light (presence or absence) on the average number of roots produced per *M. senegalensis* shoot is shown on Fig. 5.3. The type of medium (agar-gelled or liquid) on which the shoots were cultured significantly influenced ($p=0.001$) the average number of roots produced per shoot. The average number of roots produced per shoot was significantly higher in the agar-gelled medium than in the liquid medium regardless of the presence or absence of light. The average number of roots produced per shoot in the control treatment was significantly higher compared to the number of roots produced in the shoots cultured in a liquid medium. These results imply that the agar-gelled medium, in which the shoots in the control treatment were placed during the root-growth phase, influenced adventitious root formation.

The addition of a gelling agent (0.8% agar) to the rooting medium influenced the length and the appearance of the roots. When shoots were placed on an agar-gelled medium, the roots produced were shorter than those produced in a liquid medium, were whitish in colour and lacked root hairs (Plate 5.1G). The shoots placed in a liquid medium during the root-induction and root-growth stages appeared longer (length measurements not shown), were yellowish in colour and had a thick tuft of root hairs (Plate 5.1H). The absence or presence of light did not influence the appearance of the roots.

The use of a liquid medium with shoots supported by filter paper bridges can improve the formation of better quality roots, mainly because there is free diffusion of oxygen compared with agar-gelled medium (GEORGE 1996). Roots lacking root hairs may not function in water uptake and transportation upon transplantation of the plantlets to a potting mixture later (ALDERSON *et al.* 1988).

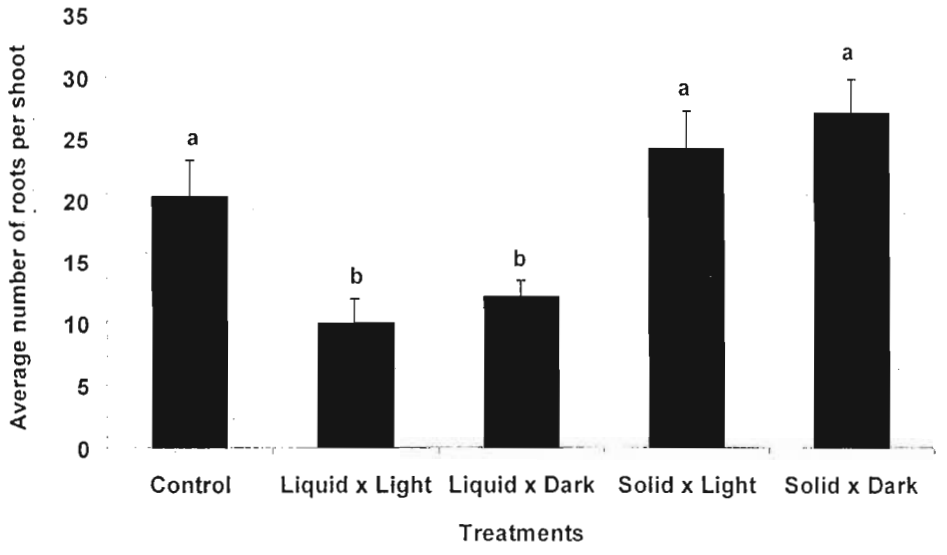


Fig. 5.3 Effect of media type (solid or liquid) and light on the average number of roots produced per *M. senegalensis* shoot, 14 days after transfer to a hormone-free medium. (Bars bearing different letters are significantly different at p=0.05).

From these results, it is apparent that the average number of roots produced per shoot in *M. senegalensis* in *vitro* shoots was influenced by the type of medium on which the shoots were cultured and not the presence or absence of light. The role of agar in plant tissue culture is assumed to be that of a neutral support (ICHIMURA and ODA 1998). However, it is known that the concentration and type of matrix influences the response of tissues cultured *in vitro* (LEE *et al.* 1986; PIERIK 1987). The beneficial effects of agar on adventitious root formation in *M. senegalensis* in *vitro* shoots could be due to the presence of a root growth stimulant. According to ICHIMURA and ODA (1998), a root growth stimulant found in some agar preparations promoted adventitious root formation in lettuce. The presence of small amounts of macro- and microelements present in agar as pollutants may also supplement the actual cultural medium, thereby influencing adventitious root formation (GEORGE 1996).

Light strongly influences organized development of tissue cultures (MURASHIGE 1974). Many workers have shown that a dark pre-treatment of shoots during the root-induction stage favours rooting (GEORGE 1996). However, the presence or absence of light in the root-induction stage did not influence adventitious root formation in *M. senegalensis in vitro* shoots (Fig. 5.3). According to GEORGE (1996), a dark treatment was not necessary to root apple shoots when optimal IBA levels were used. It can therefore be postulated that adequate concentrations of IBA were used in rooting *M. senegalensis* shoots thus negating the effects of a dark pre-treatment.

5.3.4 Effect of MS media strength

The effect of different MS (MURASHIGE and SKOOG 1962) media strengths on the average number of roots produced per shoot is shown on Fig. 5.4. Different MS media strengths had significant effects ($p<0.001$) on adventitious root formation by *M. senegalensis in vitro* shoots. High concentrations (3/4 and full strengths) significantly reduced the average number of roots produced per shoot. At 1/2 or 1/4 strength medium, the average number of roots produced per shoot was not significantly different (Fig. 5.4).

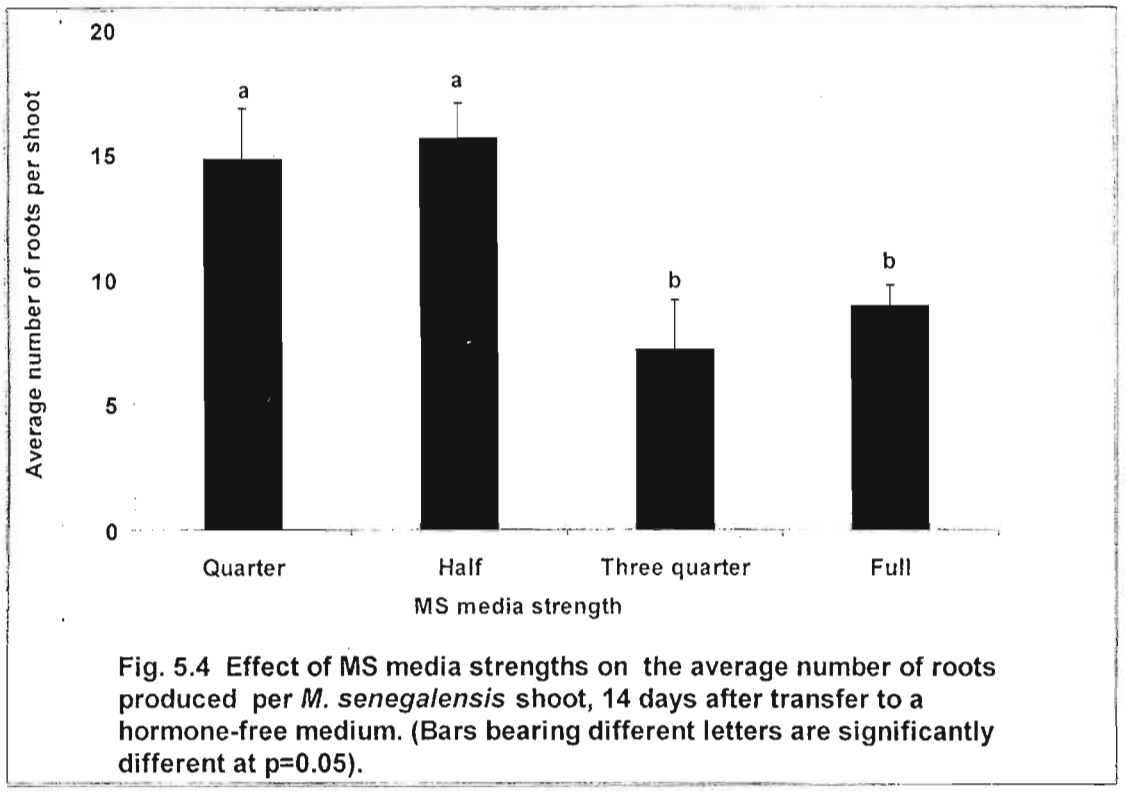


Fig. 5.4 Effect of MS media strengths on the average number of roots produced per *M. senegalensis* shoot, 14 days after transfer to a hormone-free medium. (Bars bearing different letters are significantly different at p=0.05).

The role of mineral nutrients in root formation is largely unknown (PIERIK 1987). However, root formation in many plant species was promoted at low salt concentrations (MONCOUSIN 1991a; GEORGE 1996), probably because of the reduction in nitrogen (HYNDMAN *et al.* 1982). *M. emarginata* *in vitro* shoots were for instance found to root better when placed at 1/2 rather than at full strength MS medium (RATHORE *et al.* 1992). Similar observations were made with some rose cultivars (BADZIAN *et al.* 1991).

5.3.5 Effect of activated charcoal

The effect of activated charcoal on the average number of roots produced per *M. senegalensis* shoot is shown on Fig. 5.5. The addition of activated charcoal to the rooting medium significantly ($p<0.001$) reduced the average number of roots produced per shoot. However, there were no significant differences between the average number of roots produced by shoots placed on a medium containing 5 and 10 gl^{-1} activated charcoal. The presence of 5 gl^{-1} activated charcoal promoted the production of long roots (Plate 5.1I) compared to those produced on a medium containing 10 gl^{-1} (Plate 5.1J) or the control (Plate 5.1D). The roots that were produced by shoots placed on an activated charcoal containing medium (regardless of the concentration) appeared thinner and whiter than those produced in the control treatment. The shoot bases were less swollen when shoots were placed on an activated charcoal containing medium (Plate 5.1I) than when placed on a medium lacking activated charcoal (Plate 5.1D).

Addition of activated charcoal to the rooting medium can either promote or inhibit adventitious root formation (PIERIK 1987). Several workers have established that although the presence of activated charcoal in a rooting medium may not significantly promote the percentage rooting or the number of roots produced per shoot, it may promote the quality of roots in terms of increased lengths, increased branching and development of root hairs. Such observations were made in some clones of *Quercus robur* and *Q. rubra* (SANCHEZ *et al.* 1996) and *Cercis canadensis* var *mexicana* (MACKAY *et al.* 1995).

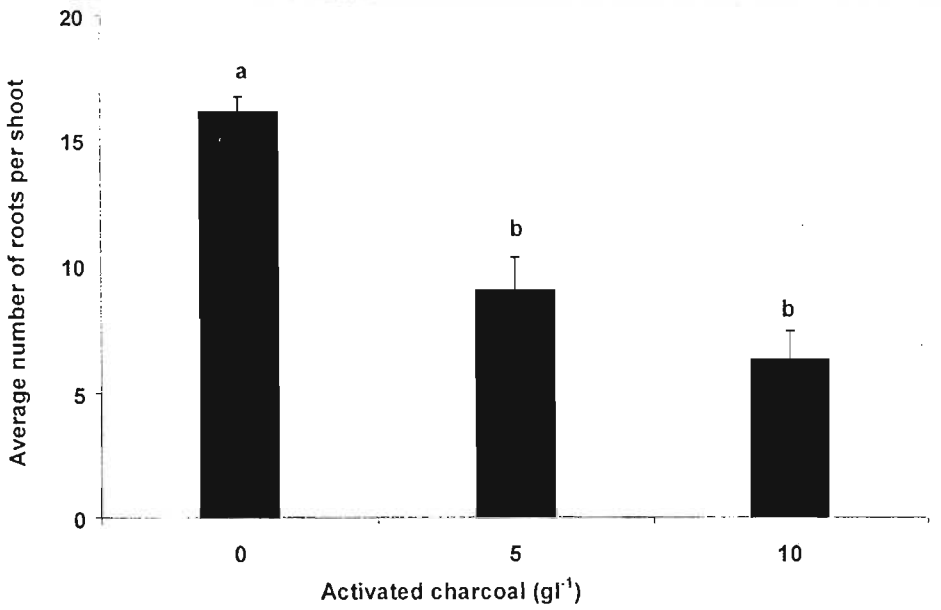


Fig. 5.5 Effect of activated charcoal on the average number of roots produced per *M. senegalensis* shoot, 14 days after transfer to a hormone-free medium. (Bars bearing different letters are significantly different at p=0.05).

The reduction on the average number of roots produced per shoot in the presence of activated charcoal in *M. senegalensis* could be attributed to its potential to adsorb growth regulators (PAN and VAN STADEN 1998). It is possible that the agar brand that was used to solidify the medium contained root growth stimulating substances, which were adsorbed by activated charcoal.

The formation of long roots in the presence of 5 gl^{-1} activated charcoal could be due to the potential of activated charcoal to adsorb phytotoxic substances that may be released by the tissues (YAM *et al.* 1990), adsorption of inhibitory components from agar (KOHLENBACH and WERNICKE 1977 or by providing added aeration (GEORGE 1996).

5.4 CONCLUSIONS

The results obtained in this part of the study have shown that adventitious root formation in *M. senegalensis in vitro* shoots is influenced by most of the factors that were investigated. The presence of auxins was found to be the most important factor because rooting was totally inhibited in the absence of IBA. The temperatures under which the shoots were rooted were also found to influence rooting. Rooting was totally inhibited at 15 °C while 28 °C was the optimal temperature. A good rooting response occurred at a wide range of sucrose concentrations (10-60 gl^{-1}) but the absence or the presence of high (100 gl^{-1}) sucrose concentrations inhibited rooting. The presence of agar in the rooting medium significantly increased the average number of roots produced per shoot. However, a liquid medium led to the production of longer roots that had a thick tuft of root hairs. Light (presence or absence) had no significant influence on rooting ability. Less concentrated MS medium (MURASHIGE and SKOOG 1962) promoted rooting while the presence of activated charcoal in the rooting medium reduced the average number of roots produced per shoot.

CHAPTER 6

IN VITRO ROOT CULTURES OF *MAYTENUS SENEGALENSIS*

6.1 INTRODUCTION

The recent past has witnessed a significant global increase in demand for medicinal plants and their extracts. This trend is expected to continue in the next 25 years and so is the pressure to produce and collect medicinal plants (CRAKER 2003). However, the development of plant-derived drugs is hampered by the continued depletion of plant genetic resources and the loss of folkloric information as many traditional cultures change their way of life (KINGHORN 1992).

Advances in plant tissue culture have the potential to alleviate the problem of supply of plant-derived compounds (KINGHORN 1992; NIGG and SEIGLER 1992). Cell, callus and organ cultures are capable of producing various types of secondary compounds (WOO *et al.* 1997; KUSAKARI *et al.* 2000; VÁZQUEZ-FLOTA and LOYOLA-VARGAS 2003). In the family Celastraceae, callus cultures of *Catha edulis* (ELHAG *et al.* 1999), *Maytenus aquifolium* (FRANCA *et al.* 1999), *M. buchananii* (KUTNEY *et al.* 1991) and *M. ilicifolia* (PEREIRA *et al.* 1993) have been shown to have the capability to produce bioactive secondary metabolites.

The culture of isolated roots enhances the production of secondary metabolites that require the presence of differentiated tissues (YAMANDA and HASHIMOTO 1988). This is because the root is a fully differentiated and an organized unit (MARÍN and MARÍN 1998). Accumulation of secondary metabolites is influenced by the manipulation of the culture environment (BALANDRIN and KLOCKE 1988). However, little is known about the effects of different factors on the growth of isolated root cultures of many woody plants (MARÍN and MARÍN 1998).

According to DODDS and ROBERTS (1985) and GEORGE (1993), attempts to initiate *in vitro* root cultures in many woody species have been unsuccessful. However, root proliferation was observed on *M. senegalensis* root explants placed on MS medium (MURASHIGE and SKOOG 1962) supplemented with 0.5 mgl^{-1} kinetin (Plate 6.1A). The establishment of an *in vitro* root culture of *M. senegalensis* would provide an alternative source of bioactive secondary metabolites and introduce the possibility of the production of such compounds on a large scale.

The objective of this part of the study was to establish *M. senegalensis in vitro* root cultures and determine the effects of some factors on root growth thereby defining conditions that allow favourable growth of the root cultures.

6.2 MATERIALS AND METHODS

6.2.1 Establishment of root culture

Seeds of *M. senegalensis* (collected in Machakos District, Eastern Province, Kenya in March 2000) were surface sterilized with 70% alcohol for 1 min and subsequently with 0.1% mercuric chloride supplemented with a few drops of Tween 20® for 12 min. The decontaminated seeds were rinsed in three changes of sterile distilled water and germinated on a salt-less medium solidified with 0.8% agar (Agar-Agar powder CP, batch 8522/151, Associated Chemical Enterprises, c.c. South Africa). The seeds were germinated in the dark at 25±2 °C. Once germinated, the seedlings were removed and placed under 16/8 h light/dark photoperiod (27 µmol m^{-2} s^{-1}) at 25±2 °C to grow. The aseptically germinated seedlings were used as a source of root explants for the induction of *in vitro* root cultures.

When the seedlings were 6-week-old, three seedlings were randomly selected and designated as genotypes A, B and C respectively. Ten mm long root segments including root tip were excised from the three genotypes and inoculated into 50 ml Erlenmeyer flasks containing 20 ml of MS (MURASHIGE and SKOOG 1962) liquid medium

supplemented with 0.1 mgl^{-1} NAA, 100 mgl^{-1} myo-inositol and 30 gl^{-1} sucrose. The pH of the medium was adjusted to 5.8 using I N KOH and 1 N HCl prior to autoclaving at 121 °C, 103.4 kPa for 20 min. The cultures were kept in the dark (wrapped with aluminium foil) at 25±2 °C and agitated at 120 RPM. To bulk up the root stocks, subcultures were made every 4 weeks for a period 3 months.

6.2. 2 Media and supplementation

In order to optimize the conditions influencing the growth of root cultures, the effects of some factors on multiplication of roots were investigated. In all the treatments, 10 mm long root segments including root tip were excised from the established root cultures (Section 6.2.1). The basal medium (BM) comprised of MS liquid medium supplemented with sucrose (30 gl^{-1}) (unless stated otherwise). Myo-inositol (100 mgl^{-1}) was also added. The pH of the medium was adjusted prior to autoclaving. The root segments were inoculated into 50 ml Erlenmeyer flasks (unless stated otherwise) containing 20 ml of BM. The cultures were kept in the dark at 25±2 °C and agitated at 120 RPM. The fresh weight (FW) of the root cultures was recorded after a period of 4 weeks. For each treatment, 5 root segments were used per flask and each treatment was replicated 5 times. All the experiments were conducted twice and the results averaged.

6.2.2.1 Effect of exogenous auxins

The effect of three auxins on multiplication of *M. senegalensis* root cultures was evaluated. Root segments were obtained from 3-month-old root cultures of *M. senegalensis* genotypes A, B and C respectively. The basal medium (BM) comprised of a liquid MS medium supplemented with 3 exogenous auxins (IAA, IBA and NAA) at various concentrations (0.01, 0.5 and 2.5 mgl^{-1}). The control treatment contained no auxins. The medium composition and the culture conditions were similar to those used during the establishment of the root cultures as outlined in Section 6.2.1.

Root segments obtained from genotype A and grown on a medium containing 0.5 mgl^{-1} NAA had the highest fresh weight. As a result, subsequent experiments were carried out using root segments obtained from genotype A and the basal medium was supplemented with NAA (0.5 mgl^{-1}).

6.2.2.2 Effect of light and media type

In order to determine the effect of light (presence or absence) and the type of medium (solid or liquid) on multiplication of *M. senegalensis* genotype A root cultures, root segments were either placed in a liquid medium or on a solid medium solidified with 0.8% agar and placed, either in the dark or at 16/8 h (light/dark) photoperiod (27 µmol $m^{-2}s^{-1}$). When a solid medium was used, the root segments were placed horizontally onto the medium (20 ml) in 90 mm plastic Petri dishes. At the end of this experiment, roots cultured in a liquid medium in the dark exhibited the best response. Consequently, root segments were cultured in a liquid medium and placed in the dark in subsequent experiments.

6.2.2.3 Effect of types of sugars

Different types of sugars viz. fructose, glucose, maltose and sucrose (30 gl^{-1}) were evaluated for their effect on growth of *M. senegalensis* genotype A root cultures. The control treatment lacked any form of sugars.

6.2.2.4 Effect of different sucrose concentrations

Different sucrose concentrations (10, 30, 60 and 100 gl^{-1}) were tested for their effect on growth of *M. senegalensis* genotype A root cultures. The control treatment contained no sucrose.

6.2.2.5 Statistical analysis

The significance of the treatment effects was determined by analysis of variance (ANOVA) using Minitab Release 12.1. Significance was accepted at p=0.05.

6.3 RESULTS AND DISCUSSION

6.3.1 Establishment of root cultures

In vitro root cultures of *M. senegalensis* genotypes A, B and C were successfully established in the presence of 0.1 mgl^{-1} NAA. However, genotypes B and C were not growing as rapidly as genotype A. As a result, it took three months before enough roots from these genotypes were available to start the subsequent experiments.

6.3.2 Effect of exogenous auxins

The effect of three exogenous auxins (IAA, IBA and NAA) on growth of *M. senegalensis* genotypes A, B and C root cultures are shown in Table 6.1. The presence of exogenous auxins had a significant effect on root multiplication ($p<0.001$). In all the genotypes, there was minimal growth of roots in the absence of auxins. In the control treatments for genotypes A (Plate 6.1B) and C (Plate 6.1D), the initial root explants elongated and produced secondary lateral roots while in the control treatment for genotype B, the initial root explants only elongated for about 5 mm, were black–tipped (root-tip necrosis) (Plate 6.1 C) and lacked secondary lateral roots.

Roots treated with various concentrations of IBA exhibited minimal growth in all three genotypes (Table 6.1). Nevertheless, the average fresh weight (FW) of the roots increased with IBA concentration. In all the genotypes, the new roots appeared thinner compared to the roots produced on media containing IAA or NAA.

Plate 6.1 Effects of exogenous auxins on root multiplication of *Maytenus senegalensis* after 4 weeks. **A**: Root proliferation in the presence of kinetin (0.5 mgl^{-1}), **B**: Extensive elongation of genotype A initial root explants in the absence of auxins, **C**: Minimal elongation of genotype B initial root explants in the absence of auxins, **D**: Moderate elongation of genotype C initial root explants in the absence of auxins, **E**: Elongation of genotype A initial root explants and extensive production of secondary lateral roots in the presence of 0.01 mgl^{-1} IBA, **F**: Production of lateral roots from all the surfaces of genotype A root explants and the production of secondary lateral roots in the presence of 2.5 mgl^{-1} IBA, **G**: Production of lateral roots that lacked secondary lateral roots by root explants of genotype B in the presence of 2.5 mgl^{-1} IBA, **H**: Moderate elongation of genotype C initial root explants in the presence of 0.01 mgl^{-1} IBA, **I**: Production of a few lateral roots that were swollen at the base by genotype C root explants in the presence of 0.5 mgl^{-1} IBA, **J**: Elongation of genotype A initial root explants and the production of a few secondary lateral roots in the presence of 0.01 mgl^{-1} IAA, **K**: Extensive production of yellow lateral roots and secondary roots by genotype A initial root explants in the presence of 0.5 mgl^{-1} IAA, **L**: Swollen genotype A initial root explants and the production of new lateral and secondary lateral roots in the presence of 2.5 mgl^{-1} IAA.

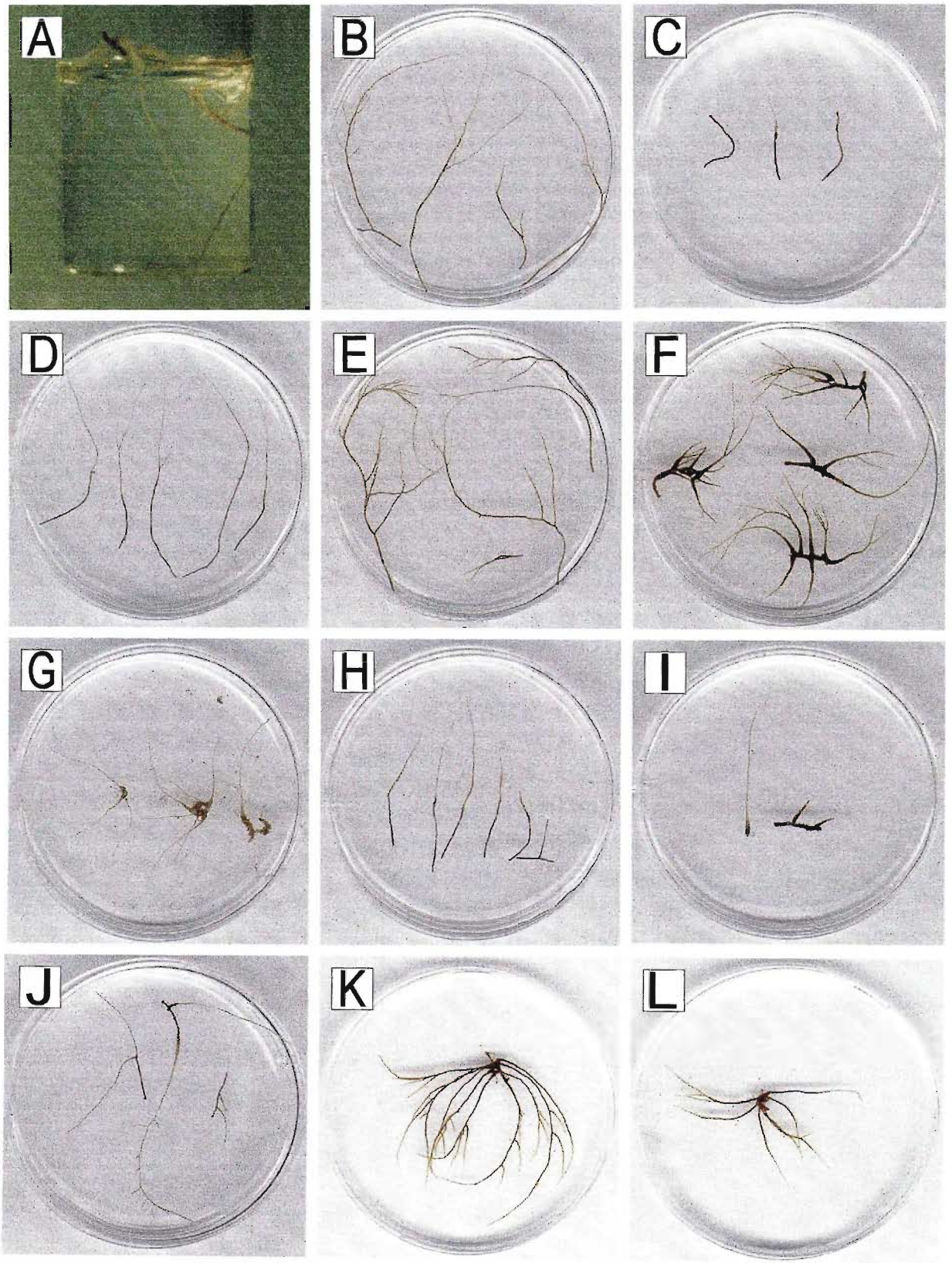

A
B
C
D
E
F
G
H
I
J
K
L

Plate 6.2 Effects of exogenous auxins on root multiplication of *Maytenus senegalensis* after 4 weeks (Contd). **A**: Swollen initial root explants and the production of a few, short (ca 5 mm long) necrotic lateral roots by genotype B root explants in the presence of 0.5 mgl^{-1} IAA, **B**: Extensive production of lateral roots with swollen root tips (shown by arrow No. i) from an extensively swollen genotype B initial root explant (shown by arrow No. ii) in the presence of 25 mgl^{-1}IAA, **C**: Moderate elongation of genotype C initial root explants in the presence of 0.01 mgl^{-1} IAA, **D**: Production of a few lateral roots that turned black by genotype C root explants in the presence of 0.5 mgl^{-1} IAA, **E**: Production of more, lateral roots that lacked secondary lateral roots by genotype C root explants in the presence of 2.5 mgl^{-1} IAA, **F**: Elongation of the initial root explants and the production of a few lateral roots, some with secondary lateral roots by genotype A initial root explants in the presence of 0.01 mgl^{-1} NAA, **G**: Extensive production of yellow lateral roots (> 50 mm long) by genotype A root explants in the presence of 0.5 mgl^{-1} NAA, **H**: Moderate production of lateral roots by genotype B root explants in the presence of 0.5 mgl^{-1} NAA, **I**: Production of short lateral roots (ca 20 mm) by genotype C root explants in the presence of 0.5 mgl^{-1} NAA, **J**: Production of thick, stumpy white lateral roots by genotype A root explants in the presence of 2.5 mgl^{-1} NAA, **K**: Extensive production of white lateral roots that were swollen at the base (shown by arrow) by genotype B root explants in the presence of 2.5 mgl^{-1} NAA, **L**: Production of tiny, stumpy lateral roots, some of which were black in colour by genotype C root explants in the presence of 2.5 mgl^{-1} NAA.

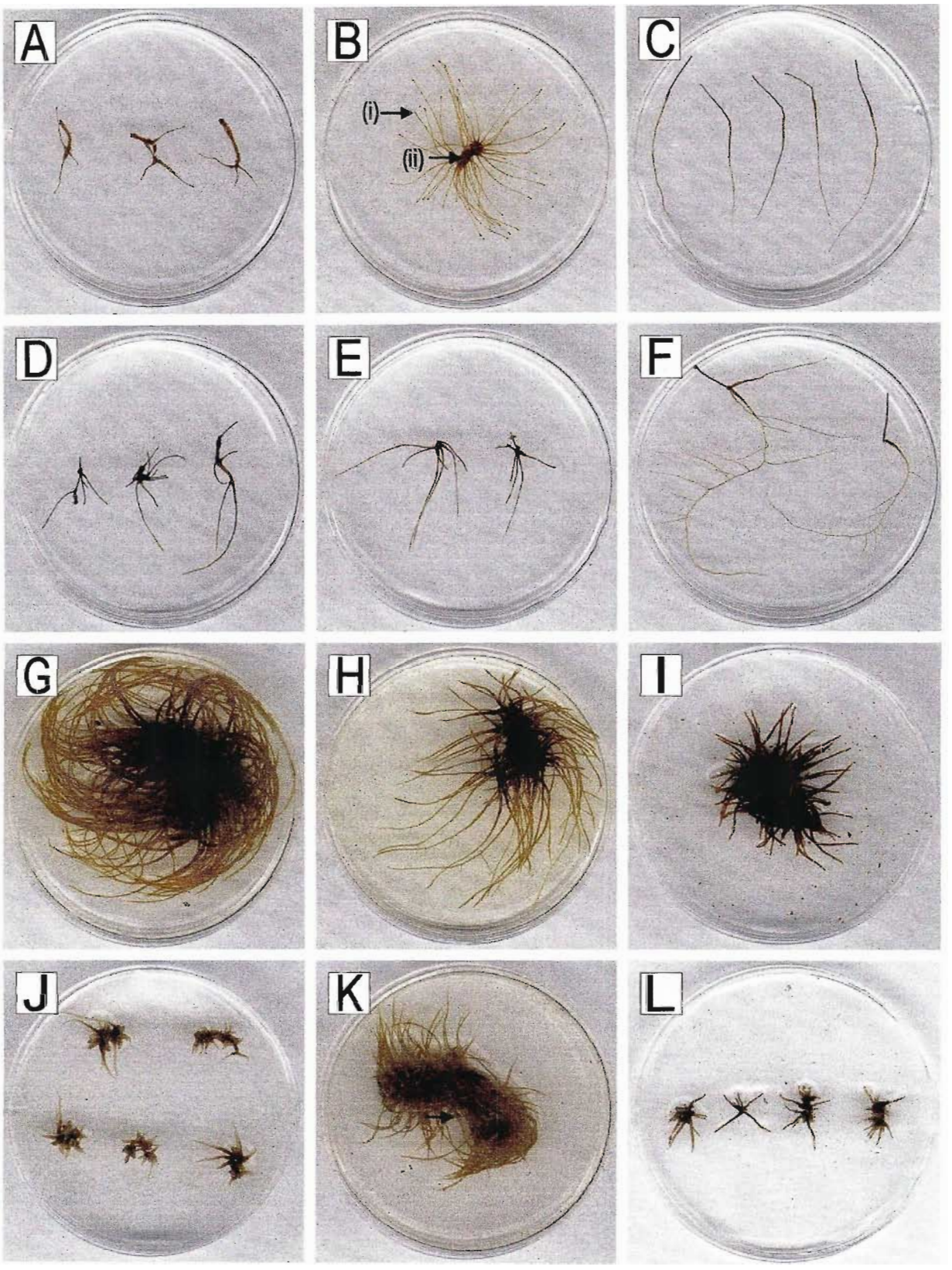

A
B
(i)
(ii)
C
D
E
F
G
H
I
J
K
L

Table 6.1 Effect of exogenous auxins on root multiplication (expressed as FW) of *Maytenus senegalensis* genotypes A, B and C after 4 weeks. (Mean FW values followed by the same letters in the same column are not significantly different at p=0.05).

Type of auxin	Concentration (mgl^{-1})	Fresh weight (g)		
		Genotype A	Genotype B	Genotype C
Control	0.00	0.01±0.00d	0.01±0.00b	0.01±0.00c
IBA	0.01	0.02±0.00d	0.01±0.00b	0.01±0.00c
	0.50	0.07±0.01cd	0.03±0.01b	0.02±0.01c
	3.00	0.15±0.04bc	0.05±0.01b	0.03±0.01c
IAA	0.01	0.02±0.01d	0.00±0.00b	0.01±0.00c
	0.50	0.18±0.03b	0.03±0.01b	0.06±0.01c
	3.00	0.22±0.04c	0.12±0.02b	0.09±0.03c
NAA	0.01	0.02±0.01d	0.01±0.00b	0.02±0.01c
	0.50	0.63±0.15a	0.45±0.16a	0.41±0.12a
	3.00	0.23±0.03b	0.41±0.11a	0.20±0.02b

In genotype A, the initial root explants placed on a medium containing 0.01 mgl^{-1} IBA elongated and produced secondary lateral roots (Plate 6.1E). However, several new lateral roots, which also produced new secondary lateral roots, were produced by explants placed on a medium containing 0.5 and 2.5 mgl^{-1} IBA (Plate 6.1F). In genotype B, there was hardly any growth of root explants placed on a medium containing 0.01 mgl^{-1} IBA. However, some of the initial root explants placed on a medium containing 0.5 or 2.5 mgl^{-1} IBA became swollen and produced a few lateral roots (Plate 6.1G). In genotype C, the initial root explants placed on a medium containing 0.01 mgl^{-1} IBA elongated to about 10 mm but no secondary lateral roots were produced (Plate 6.1H). The root explants placed on a medium containing 0.5 or 2.5 mgl^{-1} IBA were extensively swollen. Few lateral roots (ca 5mm long) lacking secondary lateral roots were produced in a few root explants (Plate 6.1I).

The average fresh weight (FW) of the roots increased with an increase on the concentration of IAA in all the genotypes (Table 6.1). In genotype A, the initial root explants placed on a medium containing 0.01 mgl^{-1} IAA were not visibly swollen. Some of the root explants elongated and produced new secondary lateral roots (Plate 6.1J). The initial root explants placed on a medium containing 0.5 mgl^{-1}appeared swollen compared to those placed on a medium containing 0.01 mgl^{-1} IAA. There were many new roots with secondary lateral roots from all the surfaces of the initial root explants (Plate 6.1K). The new lateral roots were yellow in colour. There was extensive swelling of the initial root explants placed on a medium containing 2.5 mgl^{-1} IAA. New lateral roots with secondary lateral roots were produced from all the surfaces of the initial root explants (Plate 6.1L).

In genotype B, the presence of IAA at the tested concentrations led to the production of short (ca 20 mm) roots that were swollen at the tips and lacked secondary lateral roots (Plates 6.2A and B). Extensive swelling was observed on the initial root explants placed on a medium containing 2.5 mgl^{-1} IAA (Plate 6.2 B). In genotype C, the FW and the extent of swelling exhibited by the initial root explants increased with an increase on IAA concentration. The new roots turned black in colour and lacked secondary lateral roots irrespective of the IAA concentration (Plates 6.2 C, D and E).

The addition of 0.01 mgl^{-1} NAA elicited minimal growth of roots in all the genotypes (Table 6.1) and (Plate 6.2 F). The overall maximum growth of roots was observed on medium supplemented with 0.5 mgl^{-1} NAA in all the genotypes. At this NAA concentration, new lateral roots were produced from all the surfaces of the initial root explants (Plates 6.2 G, H and I). However, the new lateral roots in genotype C (Plate 6.2I) appeared shorter than those produced by genotype A (Plate 6.2G) and B (Plate 6.2H) initial root explants. The colour of the new roots also varied. In genotype A, the new roots appeared yellowish while in genotype B, the new roots were white in colour. In genotype C, the new roots had turned black except the root tip, which appeared yellowish. When 2.5 mgl^{-1} NAA was incorporated onto the root culture medium, the new roots were shorter (ca 20 mm) compared to those produced when the medium was

supplemented with 0.5 mgl^{-1} NAA and lacked secondary lateral roots. In genotype A, the new lateral roots were thick and stumpy (Plate 6.2J). Genotype B new lateral roots were white in colour, had swollen bases and appeared longer (Plate 6.2K) than Genotype A (Plate 6.2J) and genotype C (Plates 6.2L) new lateral roots. The new lateral roots in genotype C were stumpy and black in colour except the root tip.

There is evidence that auxins either stimulate the growth of isolated roots as in *Haplopappus ravenii* (BLAKELY *et al.* 1972), *Boerhaavia diffusa* (PATHAK *et al.* 1998), *Panax ginseng* and *P. quinquefolium* (KEVERS *et al.* 1999) and *Phyllanthus caroliniensis* (CATAPAN *et al.* 2000) or inhibit it as in *Faidherbia albida* (AHÉE and DOHOUX 1994). *M. senegalensis* belongs to the former group since the application of auxins particularly IAA and NAA stimulated the growth of the root cultures. However, the presence of IAA had detrimental effects on growth of *M. senegalensis* genotype B isolated roots because it led to root-tip necrosis. Root-tip necrosis was observed in isolated roots of *Haplolappus ravenii* grown in the presence of NAA (BLAKELY *et al.* 1972).

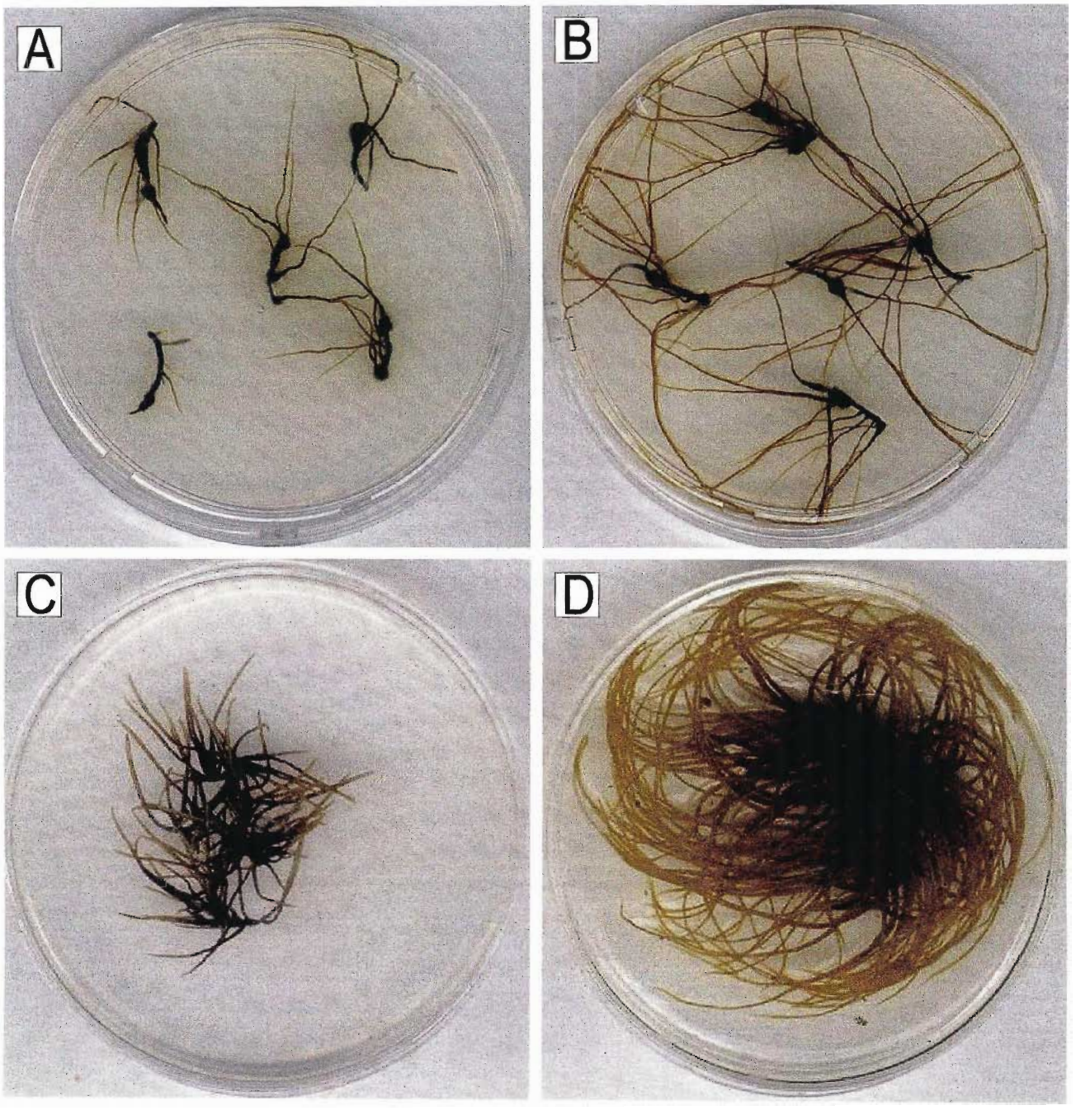
6.3.3 Effect of light and media type

The effect of light (presence or absence) and the type of medium (solid or liquid) on multiplication of *M. senegalensis* genotype A root cultures are shown in Fig. 6.1. The two factors had significant effects on the growth of the root cultures ($P<0.001$).

The addition of agar to the culture medium led to a reduction on root growth irrespective of the presence or absence of light (Plate 6.3A and B). A liquid medium on the other hand promoted the growth of roots (Plate 6.3C and D). The fresh weight (FW) of the roots cultured in the dark was higher than those cultured under light conditions regardless of the type of medium (Fig. 6.1). The overall highest fresh weight of the roots was recorded with a liquid medium placed in the dark. Darkness and a liquid medium showed the most favourable combined effects on growth of Pine roots (ULRICH 1962).

The observed inhibition of growth of the isolated roots in the presence of agar could be attributed to a reduction in salts and oxygen availability to the roots (GEORGE 1993) or the presence of root growth inhibitory substances in agar (KOHLENBACH and WERNICKE 1977, DODDS and ROBERTS 1985). The positive effects of a liquid medium could be species-specific. Rootstocks obtained from various *Prunus* cultivars exhibited poor response on a liquid medium compared to a solidified medium (MARÍN and MARÍN 1998).

Plate 6.3 The effects of light and type of medium on multiplication of *M. senegalensis* genotype A root cultures after four weeks. **A**: Root multiplication on a solid medium in the presence of light, **B**: Root multiplication on a solid medium in the dark, **C**: Root multiplication in a liquid medium in the presence of light and **D**: Root multiplication in a liquid medium in the dark.


A
B
C
D

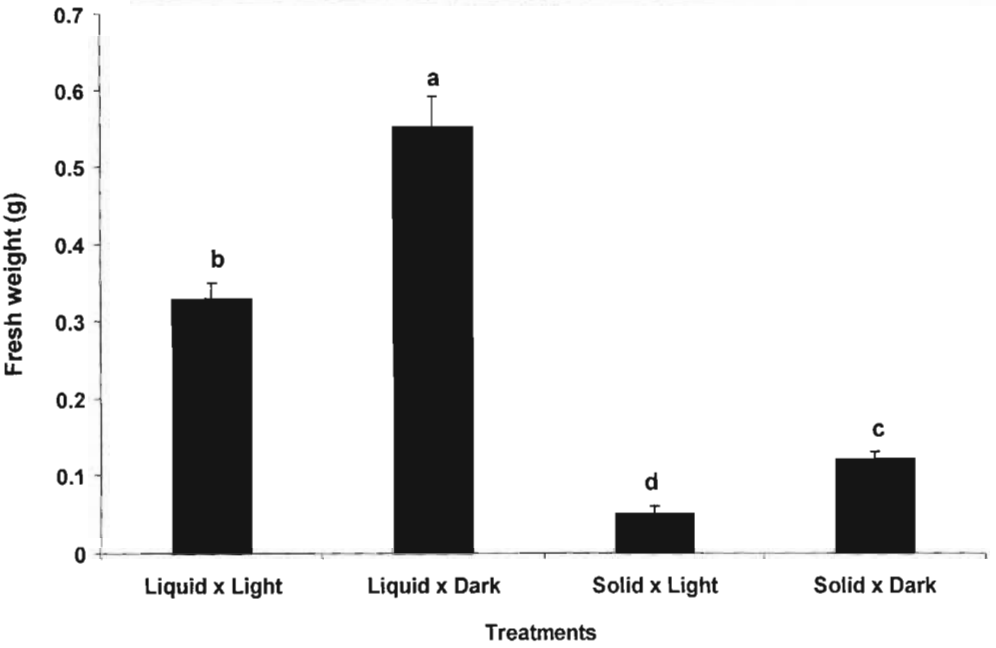


Fig. 6.1 Effect of type of medium and light on multiplication of *M. senegalensis* genotype A root cultures after 4 weeks. (Bars with different letters are significantly different at p=0.05).

6.3.4 Effect of types of sugars

The effect of different types of sugars (30 gl^{-1}) on multiplication of *M. senegalensis* genotype A root cultures are shown in Fig. 6.2. The type of sugar present in the root culture medium significantly affected the multiplication of *M. senegalensis* genotype A root cultures ($p<0.001$). The absence of any form of sugar in the medium inhibited the growth of roots.

Glucose stimulated the growth of roots as effectively as sucrose. This observation is in line with reports that both sucrose and glucose are the best sources of carbon in tissue cultures (GEORGE 1993). However, the results disagree with reports by BUTCHER and STREET (1964) to the effect that sucrose was the best sugar for the growth of isolated root cultures. Their report was based on the account of the effective translocation of sucrose to apical meristems. Although glucose promoted the growth of isolated roots in *M. senegalensis*, it was found to inhibit root growth in *Panax ginseng* and *P. quinquefolium* (KEVERS *et al.* 1999) and in *Faidherbia albida* (AHÉE and DOHOUX 1994). Sucrose is less expensive compared to glucose. Its use as a source of carbon in plant tissue cultures is thus preferred to minimize production costs (GEORGE 1993).

The addition of maltose significantly reduced root multiplication compared to glucose and sucrose. The addition of fructose was detrimental to root growth. Similar observations were made on growth of roots obtained from various *Prunus* cultivars (MARÍN and MARÍN 1998).

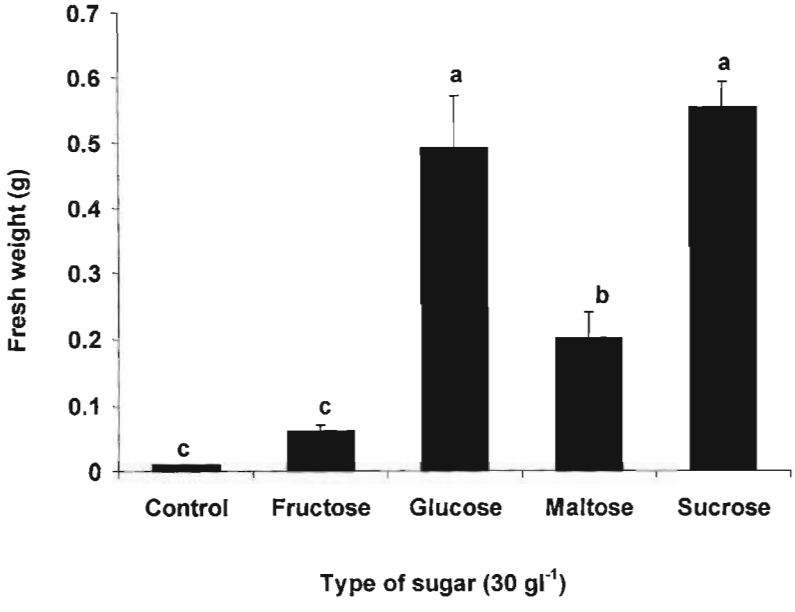


Fig. 6.2 Effect of types of sugars on multiplication of *M. senegalensis* genotype A root cultures after 4 weeks. (Bars with different letters are significantly different at p=0.05).

6.3.5 Effect of different sucrose concentrations

The presence of different sucrose concentrations in the root culture medium significantly ($p<0.001$) affected the multiplication of *M. senegalensis* genotype A root cultures as shown in Fig. 6.3. The absence of sucrose led to minimal growth of roots. An increase in sucrose concentration (10 gl^{-1}) led to an increase in root multiplication in terms of the recorded fresh weight. Roots cultured in the presence of 30 gl^{-1} sucrose had the highest FW. Higher sucrose concentration (60 and 100 gl^{-1}) led to a reduction in the fresh weight of the root cultures. The optimum sucrose concentration was therefore 30 gl^{-1}.

The addition of sucrose to a tissue culture medium is essential as it plays a critical role as an osmoticum as well as a carbon source (CONNER and FALLON 1993). Sucrose has also been reported to affect cell differentiation and to regulate gene expression in a variety of cultures (ESTRADA *et al.* 1986). Inhibition of growth at high sucrose concentrations could be attributed to a reduction in water availability to the growing roots (GEORGE 1993). Incorporation of sucrose concentrations above 4% into the root culture medium were found to inhibit the growth of isolated roots of *Faidherbia albida* (AHÉE and DOHOUX 1994).

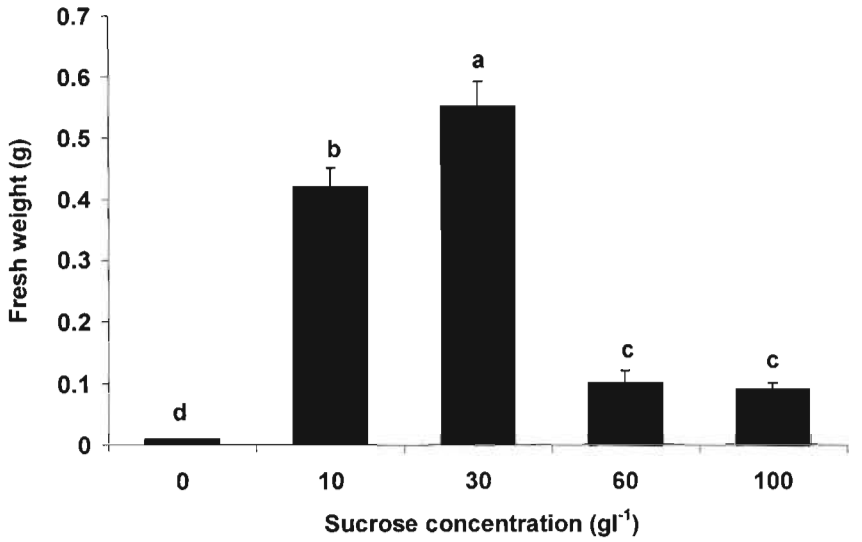


Fig. 6.3 Effect of different sucrose concentrations on multiplication of *M. senegalensis* genotype A root cultures after 4 weeks. (Bars with different letters are significantly different at p=0.05).

6.4 CONCLUSIONS

The results obtained in this part of the study showed that the addition of auxins particularly NAA (0.5 mgl^{-1}) stimulated the growth of isolated roots of *M. senegalensis* genotypes A, B and C. Low auxin concentrations (0.01 mgl^{-1}) and IBA at all the tested concentrations had minimal effects on root growth. In all the genotypes, maximum growth in terms of the recorded fresh weight (FW) was obtained when the roots were cultured on a medium supplemented with NAA (0.5 mgl^{-1}). The addition of 2.5 mgl^{-1} NAA led to the production of shorter roots compared to the roots produced in the presence of 0.5 mgl^{-1} NAA. IAA at the tested concentrations was detrimental to root growth in genotype B as it led to root-tip necrosis.

Roots grown in a liquid medium had higher fresh weights compared to roots grown on a solid medium irrespective of the presence or absence of light. Nevertheless, the roots placed in the dark had higher fresh weights compared to the roots cultured under a 16/8 h photoperiod (27 µmol m^{-2} s^{-1}), regardless of the type of medium (agar-gelled or liquid).

Glucose was as effective as sucrose in stimulating root growth while fructose was detrimental to root growth. The optimum sucrose concentration was 30 gl^{-1}. Root growth was inhibited in the in the absence of sucrose and in the presence of high sucrose concentrations (60 and 100 gl^{-1}).

CHAPTER 7

ANTIBACTERIAL ACTIVITY OF EXTRACTS PREPARED FROM *MAYTENUS SENEGALENSIS IN VITRO* ROOT CULTURES

7.1 INTRODUCTION

Various studies have established that some of the drugs that owe their origin to plants are amenable to production from plant tissue cultures (SHARP and LARSEN 1979; ROBERTS 1988). Bioactive secondary metabolites have been produced from non-transformed root cultures of various plant species such as *Datura stramonium* (MALDONADO-MENDOZA *et al.* 1992), *Digitalis lanata* (LUI and STABA 1981), *Hyoscyamus niger* (HASHIMOTO and YAMANDA 1986), *Linum flavum* (VAN UDEN *et al.* 1992) and *Papaver bracteatum* (KAMO and MAHLEBERG 1988) among others. There are no reports on previous attempts to produce antibacterial compounds or other bioactive secondary metabolites from non-transformed root cultures of any *Maytenus* species.

The basic difficulty in producing medicinal secondary metabolites from plant tissue cultures is the requirement that the material must be produced at less cost by the tissue culture than by the entire plant or by other means (synthesis or production by micro-organisms) (ROBERTS 1988). Tissue culture is a labor-intensive process. The need to maintain sterile conditions, acquisition and maintenance of fermentation facilities further aggravates the cost of production.

Many reports in the literature indicate that the production of bioactive secondary metabolites from tissue cultures is normally in very low concentrations compared to production in intact plants. However, some root cultures exhibit high levels of secondary metabolite production.

In *Salvia miltiorrhiza*, the production of tanshinones was six times more than that of the mother plants (SHIMOMURA and KITAZAWA 1991). A *Boerhaavia diffusa* root culture yielded higher levels of punarnavosid than the field grown plants (PATHAK *et al.* 1998). *M. senegalensis in vitro* root cultures were successfully established as outlined in Chapter 6. Root cultures established from genotype A in particular have been actively growing one and a half years after establishment. Root cultures are capable of synthesizing secondary metabolites similar to those produced in roots of field grown plants (LUI and STABA 1981). On the basis of the antibacterial activity exhibited by extracts prepared from *M. senegalensis* root-bark (Table 3.2), it was imperative that extracts prepared from *in vitro* root cultures be screened for antibacterial activity. The screening of plant extracts was motivated by the potential discovery of new drugs that could be developed for use later in clinical medicine. Root cultures with the potential to produce bioactive compounds provide a system for commercial production.

The aim of this part of the study was to screen extracts prepared from 8-week-old root cultures of *M. senegalensis* genotypes A, B and C for antibacterial activity. *M. senegalensis* genotype A root cultures were grown under different conditions viz. light (presence or absence), different sucrose concentrations and different MS (MURASHIGE and SKOOG 1962) media strengths and subsequently tested for antibacterial activity.

7.2 MATERIALS AND METHODS

7.2.1 Root cultures

Root cultures were established as outlined in Chapter 6 and subcultured every 4 weeks on liquid MS medium supplemented with 30 gl^{-1} sucrose, 0.1 gl^{-1} myo-inositol and 0.1 mgl^{-1} NAA. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C, 103.4 kPa for 20 min. The roots were cultured in the dark at 25±2 °C.

7.2.2 Plant material, media and supplementation

Thirty mg of 10 mm long root segments (including root tips) were inoculated into 50 ml Erlenmeyer flasks containing 20 ml of MS liquid medium supplemented with sucrose (30 gl^{-1}), myo-inostol (100 mgl^{-1}) and 0.5 mgl^{-1} NAA (BM). The pH of the medium was adjusted to 5.8 prior to autoclaving. The cultures were maintained in the dark at 25±2 °C. Five flasks were used per treatment. After a period of 8 weeks, roots obtained from different flasks of the same treatment were pooled and tested for antibacterial activity. The influence of the following factors on antibacterial activity were investigated:

1. Genotypic effects;
2. Different sucrose concentrations (10, 30, 60 and 100 gl^{-1});
3. Different MS media strengths (1/4 1/2 3/4 and full strength); and
4. Light (presence or absence).

7.2.3 Preparation of extracts

The roots harvested were cut up, placed in brown paper bags and dried at 50 °C for 72 h. The dried roots (1 g) were extracted with 10 ml methanol by sonication in an ultra sound bath for 30 min and left to macerate overnight. Extracts were then filtered using a Büchner funnel and Whatman No. 1 filter paper. The clear filtrates were placed under a stream of air to dry.

The residues were resuspended in methanol to give 100 mg residue ml^{-1}. The disc-diffusion bioassay (RASOANAIVO and RATSIMAMANGA-URVEG 1993) as outlined in Section 3.2.3, Chapter 3 was used to determine the growth inhibition caused by root culture extracts against the following bacterial strains: *Bacillus subtilis* (ATCC 4698), *Staphylococcus aureus* (ATCC 12600) (Gram-positive), *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883) (Gram-negative). Neomycin (500 μgml^{-1}) and methanol saturated discs were used as positive and negative controls respectively.

Results given are the mean inhibition zone (±S.E.) of two experiments. Only positive results are shown.

7.3 RESULTS AND DISCUSSION

Antibacterial activity was only detected against Gram-positive bacterial strains. These results were in agreement with those of the crude methanolic root-bark extracts of field grown plants (Table 3.2). However, the level of activity exhibited by the root culture extracts was about three times lower than that of root-bark obtained from field grown plants. This observation was not unusual. Various studies have shown that *in vitro* production of bioactive secondary metabolites is usually at low levels compared to the field grown plants (SHIMOMURA and KITAZAWA 1991).

7.3.1 Genotypic effects on antibacterial activity

Extracts prepared from the tested genotypes viz. A, B and C exhibited antibacterial activity (Table 7.1). The extent of antibacterial activity was the same between the 3 genotypes. From the results, it is evident that compound(s) possessing antibacterial activity are synthesized in the root cultures of the three genotypes.

Table 7.1 Genotypic effects on antibacterial activity of methanolic extracts prepared from 8-week-old *in vitro* grown root cultures of *Maytenus senegalensis*.

	Bacterial strains	
Genotype	*Bacillus subtilis*	*Staphylococcus aureus*
A	0.22±0.02	0.40±0.11
B	0.17±0.05	0.44±0.13
C	0.16±0.01	0.36±0.13

7.3.2 Effect of different sucrose concentrations on antibacterial activity

The effect of different sucrose concentrations on antibacterial activity of extracts prepared from 8-week-old *in vitro* grown cultures of genotype A are shown in Table 7.2. The extent of antibacterial activity between extracts of roots cultured on medium supplemented with 10, 30 and 60 gl^{-1} sucrose was the same. However, extracts prepared from roots cultured in the presence of 100 gl^{-1} sucrose exhibited no antibacterial activity. This implies that high concentration of sucrose inhibited the production of antibacterial compounds. High concentrations of sucrose (90 gl^{-1}) inhibited the formation of saikosaponins by *Bulpleureum falcatum* root cultures (KUSAKARI *et al.* 2000).

Table 7.2 Effect of sucrose concentrations on antibacterial activity of methanolic extracts prepared from 8-week-old *in vitro* grown root cultures of *Maytenus senegalensis* genotype A.

Sucrose concentration (gl^{-1})	Bacterial strains	
	Bacillus subtilis	*Staphylococcus aureus*
10	0.22±0.02	0.25±0.02
30	0.21±0.01	0.33±0.07
60	0.26±0.06	0.20±0.00
100	0.00±0.00	0.00±0.00

7.3.3 Effect of light on antibacterial activity

The effect of light (presence or absence) on antibacterial activity of extracts prepared from 8-week-old *in vitro* grown cultures of genotype A are shown in Table 7.3. The roots cultured under dark conditions exhibited slightly higher antibacterial activity particularly against *S. aureus* compared to the roots cultured under light conditions (16/8 h photoperiod, 27 µmol $m^{-2}s^{-1}$). These results presumably indicate that dark conditions stimulate the production of antibacterial compound(s). Light was found to suppress the production of galloyiglucoses and riccionidin A in *Rhus javanica* root cultures (TANIGUCHI *et al.* 2000)

Table 7.3 Effect of light on antibacterial activity of methanolic extracts prepared from 8-week-old *in vitro* grown root cultures of *Maytenus. senegalensis* genotype A.

	Bacterial strains	
Condition	*Bacillus subtilis*	*Staphylococcus aureus*
Light	0.21±0.01	0.38±0.14
Dark	0.25±0.01	0.45±0.14

7.3.4 Effect of MS media strengths on antibacterial activity

The effect of different MS (MURASHIGE and SKOOG 1962) media strengths on antibacterial activity of extracts prepared from *Maytenus senegalensis* genotype A isolated root cultures are shown in Table 7.4. The extent of antibacterial activity was the same between the extracts prepared from roots cultured in different media strengths. These results could mean that dilution of the macro- and micronutrients do not influence the formation of antibacterial compound(s). The concentration of the medium does influence the formation of secondary metabolites. For instance, *Cephaelis ipecacuanha* root cultures optimally produced two alkaloids viz. emetine and cephaeline in ½ strength MS media strength (SUMITA *et al.* 1991).

Table 7.4 Effect of MS media strengths on antibacterial activity of methanolic extracts prepared from 8-week-old *in vitro* grown root cultures of *Maytenus senegalensis* genotype A.

	Bacterial strains	
Media strength	*Bacillus subtilis*	*Staphylococcus aureus*
1/4	0.22±0.00	0.33±0.03
1/2	0.28±0.01	0.42±0.07
3/4	0.20±0.02	0.42±0.06
Full	0.17±0.01	0.35±0.08

7.4 CONCLUSIONS

Maytenus senegalensis in vitro grown root cultures exhibited antibacterial activity against Gram-positive bacterial strains. Nevertheless, the level of antibacterial activity was about three times lower when compared to that of extracts obtained from field grown plants. There were no major variations in the extent of antibacterial activity by some of the factors investigated viz. genotypic effects, different media strengths and the presence or absence of light. Root cultures grown in the presence of 100 gl^{-1} sucrose had no antibacterial activity.

The demonstration of antibacterial activity by *in vitro* grown root cultures emphasizes the potential of *M. senegalensis* root cultures as a source of antibacterial compounds. Although the level of activity was lower than that of field grown plants, unsustainable removal of plants parts such as the roots and the stem bark may lead to the death of the plants thereby making root cultures a potential alternative source of antibacterial compounds. There are reports that *M. senegalensis* populations are on the decline in east Africa. Consequently, an alternative source of bioactive compounds could to an extent reduce the pressure from the wild stocks.

CHAPTER 8

GENERAL CONCLUSIONS

The use of plants in traditional medicine has been in existence since time immemorial. However, the recent past has experienced a global upsurge of interest on medicinal plants. In order to meet the ever-increasing demand, a form of trade at local, regional and international levels has developed. As a result, there are reports that some of the popular medicinal plants are threatened with extinction due to overcollection. Traditional medicine preparations are mostly prepared from plant parts such as the bulbs, roots and the stem-bark, parts, which, if unsustainably removed could threaten the survival of the plant. Medicinal plants are further threatened by habitat destruction, a phenomenon that is more pronounced in the tropics. Apparently, most of the worlds' flora is located in the tropics. It is widely acknowledged that most of the present day conventional drugs owe their origin to plants. However, only a small proportion of the world flora has been studied extensively in regard to medicinal properties. There is need therefore to establish the potentials of plants especially those that are used in traditional medicine as sources of drugs. The need to isolate and identify novel antibiotics from plants is precipitated by a resurgence of bacterial strains that are resistant to the currently available antibiotics. There is also need to identify anti-inflammatory compounds that are particularly selective COX-2 inhibitors from plants.

Some of the plant species screened in this study exhibited anti-bacterial activity against Gram-positive bacterial strains. Antibacterial activity was exhibited by extracts prepared from *Conyza schimperiana*, *Galinsoga parviflora*, *Maytenus senegalensis*, *Plectranthus barbatus*, *Spiranthes mauritianum*, *Zanthoxylum chalybeum* and *Z. usambarense*. Methanolic extracts of *P. barbatus* leaves exhibited high antibacterial activity. Presence of antibacterial activity in the leaves extracts demonstrates the potentials of substituting leaves for roots and stems as a conservation option. High anti-inflammatory activity was exhibited by most of the plant species tested. Generally aqueous extracts showed lower

activity except in the case of *Croton macrostachyus*, *Maytenus senegalensis*, *Zanthoxylum chalybeum* and *Z. usambarense*. Due to unavailability of adequate plant material, these plants were not screened against COX-2 enzyme, which is one of the isoforms of COX. It has been established that compounds exhibiting higher inhibition of COX-2 compared to COX-1 are likely to have minimal unwanted side effects. These results form a good basis for selection of candidate plants species for further pharmacological studies and conservation purposes.

Although the details were not provided in this study, attempts to isolate antibacterial compound(s) from a methanolic extract of *M. senegalensis* root-bark were made using bioassay guided fractionation. Extensive streaking occurred when the fractions were spotted on a TLC plate, making the identification of fractions with similar chemical profiles difficulty. Activity of one of the bioactive fractions was lost during the purification process presumably due to difficulties encountered while eluting the compound(s) from silica. Further attempts to isolate and identify the antibacterial compound(s) should therefore be made in future. This was not feasible in this study due to time constraints.

Using 6-week-old *in vitro* germinated seedlings as a source of explants, an efficient micropropagation protocol for *Maytenus senegalensis* was established. Nodal explants were the only ones that produced multiple shoots. Root explants placed on a medium containing kinetin (0.5 mgl^{-1}) produced new roots. The presence of cytokinins was a prerequisite for shoot induction and growth. BA was superior to kinetin in terms of the average number of shoots produced per explant. However, higher BA concentrations (1.5 and 3.0 mgl^{-1}) promoted the formation of stunted shoots (ca 2 mm long). Kinetin at higher concentrations (3 mgl^{-1}) promoted the average length of shoots. There were no significant interactions between BA and auxins (IAA and IBA) at low concentrations (0.1, 0.5 and 1.0 mgl^{-1}) on either the average number of shoots produced per explant or the average length of shoots. BA was the only plant growth regulator that had significant main effect on both the average number of shoots produced per explant and the average length of shoots. Shoots were multiplied in the presence of BA (0.5 mgl^{-1}) alone.

Attempts to root the *M. senegalensis in vitro* grown shoots were initially difficult. However, *in vitro* rooting was achieved by pulse treating 8-week-old shoots in the dark for 120 h in a liquid MS medium containing 25 mgl^{-1} IBA at 28 °C. The pulse treated shoots were then transferred to a hormone free solid medium at 12/12 h photoperiod for the roots to grow. In an attempt to optimize the IBA concentration and the period of pulse treatment during the root induction phase, the results showed that each of the two factors had a significant effect on their own and there was a significant interaction between them. The rooted shoots were successfully acclimatized.

An investigation on the effects of various factors on adventitious root formation of 8-week-old *M. senegalensis* shoots showed that auxins are a prerequisite for rooting. No rooting occurred in the absence of auxins. Temperatures at which the *in vitro* shoots were rooted significantly influenced the formation of adventitious roots. Rooting was totally inhibited at 15 °C. Optimal rooting occurred at 28 °C. When the shoots were rooted at 35 °C, root emergence occurred 2-3 days after transfer to a hormone free medium compared to 4-5 days at 23 and 28 °C respectively. Shoot necrosis occurred when the shoots were rooted at 35 °C. A wide range of sucrose concentrations (10-60 gl^{-1}) was optimal for adventitious root formation. However, the absence of sucrose or the presence of high sucrose concentrations (100 gl^{-1}) significantly reduced the average number of roots produced per shoot. The presence or absence of light during the root induction stage had no effect on adventitious root formation. However, shoots placed on a solid medium produced a significantly higher number of roots compared to the shoots placed on filter paper bridges in a liquid medium. Nevertheless, shoots rooted on a liquid medium produced longer roots that had root hairs. The presence of activated charcoal (AC) in the hormone-free, root growth medium significantly reduced the number of roots produced per shoot. However, the roots produced in the presence of activated charcoal were longer and appeared whiter than those produced in the absence of charcoal.. These results confirmed that adventitious root formation in *M. senegalensis in vitro* grown shoots is influenced by a variety of factors. It is known that the conditions under which shoots are rooted may influence acclimatization. The influence of these factors on the survival of the rooted shoots under *ex vitro* conditions should therefore be

investigated. This was the first attempt to investigate the influence of various factors (physiological and environmental) on adventitious root formation in any *Maytenus* species

In vitro root cultures are potential alternative sources of bioactive secondary metabolites. Although there were no previous reports on attempts to establish *in vitro* roots cultures of any *Maytenus* species, *M. senegalensis in vitro* root cultures were successfully established using 6-week-old *in vitro* germinated seedlings as a source of root explants. The type and concentration of auxins was found to influence the growth of root cultures. The absence of auxins and the presence of IBA at the tested concentrations (0.01, 0.5 and 2.5 mgl^{-1}) had minimal effects on growth of isolated root cultures of the three genotypes investigated. Low auxins concentration (0.01 mgl^{-1}) elicited minimal growth of root cultures in all the genotypes. Root tip necrosis was observed in genotype B root cultures. However, this detrimental effect was more pronounced in the presence of IAA. The overall maximum growth of root cultures was on a medium supplemented with NAA (0.5 mgl^{-1}. Incorporation of 2.5 mgl^{-1} NAA in the root culture medium led to the production of short stumpy roots in genotype A, white roots with swollen bases in genotype B and short black roots in genotype C. Optimal growth of root cultures occurred when the root culture medium was supplemented with 30 gl^{-1} sucrose. The absence of sucrose totally inhibited root growth while high sucrose concentrations (60 and 100 gl^{-1}) significantly reduced root multiplication. The type of sugar (30 g^{-1}) in the root culture medium was found to significantly influence the growth of roots. The growth of root cultures was highest in the presence of sucrose and glucose compared to maltose or fructose. Light (presence or absence) and the type of medium (agar-gelled or liquid) significantly influenced the growth of root cultures. A liquid medium and dark conditions were the most favourable combinations for growth of *Maytenus senegalensis* genotype A root cultures.

Methanolic extracts prepared from 8-week-old *in vitro* root cultures exhibited antibacterial activity against Gram-positive bacterial strains. The extent of antibacterial activity was however lower than that of extracts prepared from root-bark obtained from

field grown plants. This observation was not unusual. Many workers have reported that tissue cultures produce lower levels of bioactive compounds compared to field grown plants. Nevertheless, *Maytenus senegalensis* populations are on the decline in east African countries. These results therefore form a good basis for further pharmacological and phytochemical research in that an alternative source of antibacterial compounds such as *in vitro* root cultures could to an extent reduce the pressure from wild sources.

REFERENCES

ABEBE D (1987). Plants in the health care delivery systems of Africa. In: Leeuwenberg, AJM (compiler). Medicinal and Poisonous Plants of the Tropics. Proceedings of symposium 5-35 of the 14th International Botanical Congress, Berlin, 25th July-1st August 1987.

ABEBE D and AYEHU A (1993). Medicinal Plants and Enigmatic Health Practices in Ethiopia. Addis Ababa, Ethiopia.

ABO EL-NIL MM (1997). Tissue culture of native plants in the developing countries. Acta Horticulturae **447**: 507-513.

ADDAE-MENSAH I (1992). Towards a Rational Scientific Basis for Herbal Medicine - A Phytochemists Two Decade Contribution. Ghana Universities Press, Accra.

AHEE J and DOHOUX E (1994). Root culturing of *Faidherbia=Acacia albida* as a source of explants for shoot regeneration. Plant Cell, Tissue and Organ Culture **36**: 219-225.

AKERELE O (1984). WHO'S traditional medicine programme: progress and perspectives. WHO Chronicle **38**: 76-81.

AKERELE O (1991). Medicinal plants: policies and priorities. In: Akerele O, Heywood V and Synge H (eds). The conservation of medicinal plants. Proceedings of an International Consultation, 21st –27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

AKETCH CA (1992). A preliminary survey of the conservation status of some plant species in Kenya. In: Edwards S and Asfaw Z (eds). The status of some plant resources in parts of tropical Africa. Botany 2000: East and Central Africa. NAPRECA monograph series No. 2. NAPRECA, Addis Ababa University, Addis Ababa.

ALDERSON PG, McKINLESS J and RICE RD (1988). Rooting of cultured Rose shoots. Acta Horticulturae **226**: 175-179.

ALFERMANN AW and PETERSEN M (1995). Natural product formation by plant cell biotechnology. Plant Cell, Tissue and Organ Culture **43**: 199-205.

ALVARENGA NL, VELÁZQUEZ CA, GÓMEZ R, CANELA NJ, BAZZOCCHI IL, FERRO EA (1999). A new antibiotic nortriterpene quinone methide from *Maytenus catingarum*. Journal of Natural Products **62**: 750-751.

ANAND A, SRINVASA C and BALAKRISHNA (1999). *In vitro* propagation of *Syzgium travancoricum* Gamble – an endangered tree species. Plant Cell, Tissue and Organ Culture **56**: 59-63.

ARCHER RH and JORDAAN M (2000). Celastraceae. In: Leistner OA (ed). Seed plants of southern Africa: Families and genera. Sterelitzia, National Botanical Institute, Pretoria.

AREGBEYEN JBO (1996). Traditional herbal medicine for sustainable PHC. Indigenous Knowledge and Development Monitor **4**: 14-15.

AROCKIASAMY S and IGNACIMUTHU S (1998). Plant regeneration from mature leaves and roots of *Eryngium foetidum* L., a food-flavouring agent. Current Science **75**: 664-666.

ATTISO MA (1983). Phytopharmacology and phytotherapy. In: Bannerman RH, Burton, J and Wen-Chie C (eds). Traditional medicine and health care coverage. World Health Organization, Geneva.

BADZIAN T, HENNEEN GR and FOTYMA-KERN J (1991). *In vitro* rooting of clonal propagated miniature rose cultivars. Acta Horticulturae **289**: 329-330.

BALANDRIN MF and KLOCKE JA (1988). Medicinal, aromatic and industrial materials from plants. In: Bajaj YPS (Ed.). Biotechnology in Agriculture and Forestry. Vol. 4. Medicinal and Aromatic Plants. Springer-Verlag, Berlin. Pp 3-36.

BALANDRIN MF, KINGHORN AD and FARNWORTH NR (1993). Plant-derived natural products in drug discovery and development t: An overview. In: Kinghorn AD and Balandrin MF (Eds.). Human Medicinal Agents from Plants. American Chemical Society, Washington DC. Pp 2-12.

BANNERMAN RH (1983). The role of traditional medicine in primary health care. Phytopharmacology and phytotherapy. In: Bannerman RH, Burton J and Wen-Chie C (Eds.). Traditional Medicine and Health Care Coverage. World Health Organization, Geneva. Pp 318-327.

BAQUAR SR (1992). Economic importance of yams (*Dioscorea* spp.) and their natural products with special reference to West Africa. In: Edwards S and Asfaw Z (eds). The status of some plant resources in parts of tropical Africa. Botany 2000: East and Central Africa. NAPRECA monograph series No. 2. NAPRECA, Addis Ababa University, Addis Ababa. Pp19-33.

BATANOUNY KH (1999). The wild medicinal plants in North Africa: history and present status. Acta Horticulturae **500**:183-188.

BATLLE A and ALDRUFEU A (1987). Some factors influencing *Nephrolepis* accilmatization. Acta Horticulturae **212**: 367-370.

BEENA MR, MARTIN KP, KIRTI PB and HARIHARAN M (2003). Rapid *in vitro* propagation of medicinally important *Ceropegia candelabrum*. Plant Cell, Tissue and Organ Culture. **72**: 285-289.

BEENTJE HJ (1994). Kenya Trees, Shrubs and Lianas. National Museums of Kenya, Nairobi, Kenya.

BENGOCHEA T (1983). Culture of ornamental trees. In: Dodds JH (Ed.). Tissue Culture of Trees. Kroom Helm Ltd, Beckenham.

BLAKELY LM, RODAWAY SJ, HOLLEN LB and CROKER SG (1972). Control and kinetics of branch root formation in cultured root segments of *Haplopappus ravenii*. Plant Physiology **50**: 35-42.

BLOCK E (1986). Antithrombotic agent of garlic: A lesson from 500 years of folk medicine. In: Steiner RP (ed). Folk medicine: The Art and the Science. American Chemical Society, Washington DC.

BONATI A (1991). Industry and the conservation of medicinal plants. In: Akerele O, Heywood V and Synge H (eds). The conservation of medicinal plants. Proceedings of an International Consultation, 21st –27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

BROWN MRW, GILBERT P, KLEMPERER RMM (1979). Influence of the bacterial cell envelope on combined antibiotic action. In: Williams JD (Ed.). Antibiotic Interactions. Academic Press, London.

BUCKMAN R and SABBAGH K (1993). Magic or Medicine: An Investigation of Healing and Healers. M. Macmillan, London.

BURTON GR and ENGELKIRK PG (1995). Microbiology for the Health Sciences. 5th Edition. Lippincott-Raven Publishers, Philadelphia.

BUTCHER DN and STREET HE (1964). Excised root culture. The Botanical Review **30**: 513-586.

BUTENKO RG (1968). Plant Tissue Culture and Plant Morphogenesis. Israel Program for Scientific Translation Ltd, Jerusalem.

CATAPAN E, OTUKI MF and VIANA A (2000). *In vitro* cultures of *Phyllanthus caroliniensis* (Euphorbiaceae). Plant Cell, Organ and Tissue Culture **62**: 195-202.

CHAVEZ H, RODRIGUEZ G, ESTEVVEZ BA, RAVELO AG, ESTEVEZ-REYES R, GONZALEZ AG, FDEZ-PUENTE JL and GARCIA-GRAVALOS D (2000). Macrocarpins A-D, new cytotoxic nor triterpenes from *Maytenus macrocarpa*. Bioorganic and Medicinal Chemistry Letters **10**: 759-762.

CHÉE R and POOL RM (1988). Sucrose and NAA influence growth of subcultured shoots and *in vitro* production of roots in *Vitis*. Horticultural Science **23**: 776.

CHUKWUJEKWU JC, FENNELL CW and VAN STADEN J (2002). Optimization of the tissue culture protocol for the endangered *Aloe polyphylla*. South African Journal of Botany **68**: 424-429.

CLARK AM and HUFFORD CD (1993). Discovery and development of novel prototype antibiotics fro opportunistic infections related to Acquired Immunodeficiency Syndrome. In: Kinghorn AD and Balandrin MF (Eds.). Human Medicinal Agents from Plants. American Chemical Society, Washington DC. Pp 228-241.

COLE MD (1992). The significance of the terpenoids in the Labiatae. In: Harley RM and Reynolds T (Eds.). Advances in Labiatae Science, Royal Botanic Gardens, Kew.

COLLINS HA and EDWARDS S (1998). Plant Cell Culture. Bios Scientific Publishers Ltd, Oxford.

CONNER AJ and FALLOON PG (1993). Osmotic versus nutritional effects when rooting *in vitro* asparagus minicrowns on high sucrose media. Plant Science **89**: 101-106.

CONSTANTIN MJ (1978). Historical perspectives and introduction. In: Hughes KW, Henke R and CONSTANATIN M (Eds.). Propagation of Higher Plants Through Tissue Culture – A Bridge Between Research and Application. Proceedings of an International Symposium held at the University of Tennessee, Knoxville. 16-19th April, 1978.

CORDEIRO PJM, VILEGAS JHY and LANCAS FM (1999). HRGC-MS analysis of terpenoids from *Maytenus ilicifolia* and *Maytenus aquifolium* ('Espinheira Santa"). Journal of Brazillian Chemical Society **10**: 523-526.

COTTON CM (1996). Ethnobotany: Principles and Applications. John Wiley and Sons, Chichester.

COWAN MM (1999). Plant products as antimicrobial agents. Clinical Microbiology Review **12**: 564-582.

COX PA (1994). The ethnobotanical approach to drug discovery: strengths and limitations. In: Prance GT, Chadwick DJ and Marsh J (Eds.). Ethnobotany and the search for new drugs. Ciba Foundation Symposium 185. John Wiley and Sons, Chichester

CRAKER LE (1999). Trends in medicinal and aromatic plant production in the United States. Acta Horticulturae **502**: 71-75.

CRAKER LE (2003). Production and demand-A view to the future of MAP. Acta Horticulturae **597**: 71-75.

CUNNINGHAM AB (1991). Development of a conservation policy on commercially exploited medicinal plants: A case study from southern Africa. In: Akerele O, Heywood V and Synge H (eds). The conservation of medicinal plants. Proceedings of an International Consultation, 21st –27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

DAVIES FT and HARTMANN HT (1988). The physiological basis of adventitious root formation. Acta Horticulturae **227**: 113-120.

DE FOSSARD RA (1977). Tissue culture in horticulture – a perspective. Acta Horticulturae **78**: 455-459.

DEBERGH P and MAENE LJ (1981). A scheme for commercial propagation of ornamental plants by tissue culture. Scientia Horticulturae **14**: 335-345.

DE KLERK GJ (2002). Rooting of microcuttings: theory and practice. *In Vitro* Cellular and Developmental Biology-Plant. **38**: 415-422.

DHAR U and UPRETI J (1999). *In vitro* regeneration of mature leguminous liana (*Bauhinia vahlii* Wight and Arnott). Plant Cell Reports **18**: 664-669.

DHARANI N (2002). A Field Guide to Common Trees and Shrubs of East Africa. Struik Publishers, Cape Town.

DODDS JH (1983). Introduction. In: Dodds JH (Ed.). Tissue Culture of Trees. Kroom Helm Ltd, Beckenham.

DODDS JH and ROBERTS LW (1985). Experiments in Plant Tissue Culture. 2nd Edition. Cambridge University Press, Cambridge.

DREWES FE and VAN STADEN J (1995). Initiation of and solasodine production in hairy root cultures of *Solanum mauritianum* Scop. Plant Growth Regulation **17**: 27-31.

DURAN-VILA N, GOGORCENAY, ORTEGA V, ORTIZ J and NAVARRO L (1992). Morphogenesis and tissue culture of sweet orange (*Citrus sinensis* (L.) Osb.): effect of temperature and photosynthetic radiation. Plant Cell, Tissue and Organ Culture **29**: 11-18.

DURZAN DJ (1988). Rooting in woody perennials: problems and opportunities with somatic embryos and artificial seeds. Acta Horticulturae **227**: 121-125.

EL TAHIR A, IBRAHIM AM, SATTI GM, THEANDER TG, KHARAZMI A and KHALID SA (1998). The potential antileishmanial activity of some Sudanese medicinal plants. Phytotherapy Research **12**: 576-579.

EL TAHIR A, SATTI GM, and KHALID SA (1999). Antiplasmodial activity of selected Sudanese medicinal plants with emphasis on *Maytenus senegalensis*. Journal of Ethnopharmacology **64**: 227-233.

EL TAHIR A, SATTI GM and KHALID SA (2001). A novel antiplasmodial activity of pristimerin isolated from *Maytenus senegalensis* (Lam.) Exell. Journal of the Saudi Chemical Society **5**: 157-164.

ELHAG H, MOSSA JS and EL-OLEMY ME (1999). Antimicrobial and cytotoxicity activity of the extracts of Khat callus. In: Janick J (Ed.). Perspectives on New Crops and New Uses. ASHS Press, Alexandaria, VA. Pp 463-470.

ELHAG HM (1991). *In vitro* propagation of *Catha edulis*. Horticultural Science **26**: 212

ENWEREJI E (1999). Views on tuberculosis among the Igbo of Nigeria. Indigenous Knowledge and Development Monitor **7**: 3-5.

ERDELEN WR, ADMIHARDJA K, MOEDSDARARSONO H and SIDIK I (1999). Biodiversity, traditional medicine and the sustainable use of indigenous medicinal plants in Indonesia. Indigenous Knowledge and Development Monitor **7**: 3-6.

ESTRADA R, TOVAR P, DODDS JH (1986). Induction of *in vitro* tubers in a broad range of potato genotypes. Plant Cell, Tissue and Organ Culture **7**: 3-10.

ETKIN NL (1998). Indigenous patterns of conserving biodiversity: pharmacologic implications. Journal of Ethnopharmacology **63**: 233-245.

EVANS WC (1996). Trease and Evans' Pharmacognosy 14th Edition. Saunders W.B Company Ltd. London.

FABRY W, OKEMO PO and ANSORG R (1998). Antibacterial activity of east African plants. Journal of Ethnopharmacology **60**: 79-84.

FARNSWORTH NR (1983). The NAPRALERT database as an information source for application to traditional medicine. In: Bannerman RH, Burton, J and Wen-Chie C (Eds). Traditional medicine and health care coverage. World Health Organization, Geneva.

FARNSWORTH NR, AKERELE O, BINGEL AS, SOEJARTO DD AND GUO Z (1985). Medicinal plants in therapy. Bulletin of the World Health Organization **64**: 965-981.

FARNSWORTH NR and SOEJARTO DD (1991). Global importance of medicinal plants. In: Akerele O, Heywood V and Synge H (Eds). The Conservation of Medicinal Plants. Proceedings of an International Consultation, 21-27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

FARNSWORTH NR (1994). Ethnopharmacology and drug development. In: Prance GT, Chadwick DJ and Marsh J (Eds.). Ethnobotany and the search for new drugs. Ciba Foundation Symposium 185. John Wiley and Sons, Chichester. Pp 42-59

FRANCA SC, DUARTE IB, PEREIRA AMS, CARVALHO D and QUEIROZ MEC (1999). Triterpenes and phenolics in callus of *Maytenus aquifolium* Mart. Acta Horticulturae **502**: 363-368.

FRATKIN E (1996). Traditional Medicine and concepts of healing among Samburu pastoralists of Kenya. Journal of Ethnobiology **16**: 63-97.

FRIDBORG G and ERIKSSON T (1975). Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiologia Plantarum **34**: 306-308.

FRIDBORG G, PEDERSON M, LANDSTROM L and ERIKSSON T (1978). The effect of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morpphogensis. Physiologia Plantarum **43**: 104-106.

GACHATHI NF (1989) Kikuyu Botanical Dictionary of Plant Names and Uses. FN Gachathi, Nairobi.

GAKUNJU DMN, MBERU EK, DOSSAJI SF, GRAY AI, WAIGH RD, WATERMAN PG and WATKINS WM. (1995). Potent antimalarial activity of the alkaloid nitidine, isolated from a Kenyan herbal remedy. Antimicrobial Agents and Chemotherapy **39**: 2606-2909.

GAMBORG OL (1975). Callus and cell culture. In: Gamborg OL and Wetter LR: (Eds). Plant Tissue Culture Methods. National Research Council of Canada, Saskatoon.

GARTON S, READ PE and FARNHAM RS (1983). Effect of stock plant nutrition and macro and micropropagability of *Salix*. Acta Horticulturae **131**: 141-151.

GELFAND M, MAVI S, DRUMMOND RB and NDEMERA B (985) The traditional medical practitioner in Zimbabwe. Mambo Press, Gweru.

GEORGE EF (1993). Plant Propagation by Tissue Culture. Part 1: The Technology. Exegetics Ltd, Edington.

GEORGE EF (1996). Plant Propagation by Tissue Culture. Part 2: In Practise. Exegetics Ltd, Edington.

GESSLER MC, NKUNYA MHH, MWASUMBI LB, HEINRICH M and TANNER M (1994). Screening Tanzanian medicinal plants for antimalarial activity. Acta Tropica **5**: 65-77.

GESSLER MC, TANNER M, CHOLLET J, NKUNYA MHH and HEINRICH M (1995). Tanzanian medicinal plants used traditionally for the treatment of malaria: *in vivo* antimalarial and *in vitro* cytotoxic activities. Phytotherapy Research **9**: 504-508.

GIBBONS S, LEIMKUGEL J OLUWATUYI M and HEINRICH M (2003). Activity of *Zanthoxylum clava-herculis* extracts against multi-drug resistant methicillin-resistant *Staphylococcus aureus* (mdr-MRSA). Phytotherapy Research **13**: 274-275.

GILES KL and SONGSTAND DD (1990). Plant tissue culture in the twenty first century. In: Bhojwani SS (Ed.) Plant tissue culture: Applications and Limitations. Elsevier Science Publishing Company INC. New York.

GONZÀLEZ AG, ALVARENGA NL, RAVELO AG, JIMENEZ IA, BAZZOCCHI IL, CANELA NJ, MOUJIR LM (1996). Antibiotic phenol nor-triterpenes from *Maytenus canariensis*. Phytochemistry **43**:129-132.

GONZÀLEZ AG, ALVARENGA NL, BAZZOCCHI IL, RAVELO AG, MOUJIR L (1998). A new bioactive norquinone-methide triterpene from *Maytenus scutiodes*. Planta Medica **64**:769-771.

GONZÀLEZ FG, PORTELA TY, STIPP EJ and DI STASI LC (2001). Antiulcerogenic and analgesic effects of *Maytenus aquifolium*, *Sorocea bomplandii* and *Zolernia ilicifolia*. Journal of Ethnopharmacology **77**: 41-47.

GOOD CM and KIMANI VN (1980). Urban traditional medicine: a Nairobi case study. The East African Medical Journal **57**: 301-316.

GOVIL S and GUPTA SC (1997). Commercialization of plant tissue culture in India. Plant Cell, Tissue and Organ Culture **51**: 65-73.

GREENWOOD D (1992). Antimicrobial agents. In: Greenwood D, Slack R and Peitheren J (Eds.). Medical Microbiology – A guide to Microbial Infections, Pathogenesis, Immunity, Laboratory Diagnosis and Control. 14th Edition. Churchill and Livingston, Edinburgh. Pp 67-77.

HAMANN O (1991). The joint IUCN-WWF plants conservation programme and its interest in medicinal plants. In: Akerele O, Heywood V and Synge H (Eds). The conservation of medicinal plants. Proceedings of an International Consultation, 21st - 27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

HAMBURGER MO and CORDELL GA (1987). A direct bioautographic TLC assay for compounds possessing antibacterial activity. Journal of Natural products **50**:19-22.

HARBONE JB and BAXTER H (1993). Phytochemical Dictionary and Handbook. Taylor and Francis Ltd, London.

HARRY IS and THORPE TA (1994). *In vitro* culture of forest trees. In: Vasil IK and Thorpe TA (Eds.). Plant Cell and Tissue Culture. Kluwer Academic Publishers, Dordrecht.

HASHIMOTO T and YAMANDA Y (1986). Hyoscyamine 6β-Hydroxylase, a 2-Oxoglutarate-dependent dioxygenase, in alkaloid-producing root cultures. Plant Physiology **81**: 619-625.

HEDBERG I (1987). Research on medicinal and poisonous plants of the tropics-past, present and future. In: Leeuwenberg AJM (compiler). Medicinal and poisonous plants of the tropics. Proceedings of symposium 5-35 of the 14th International Botanical Congress, Berlin, 25th July-1st August 1987.

HEDBERG I and STAUGARD F (1989). Traditional Medicinal Plants: Traditional Medicine in Botswana. Ipeleng, Gaborone.

HERRERA RM, [EREZ M, MARTIN-HERRERA DA, LOPEZ-GARCIA R RABANAL RM, ARIAS A (1996). Antimicrobial activity of extracts from plants endemic to the Canary Islands. Phytotherapy Research **10**: 364-366.

HEYWOOD V (1978). Flowering Plants of the World. Oxford University Press, Oxford.

HEYWOOD V (1999). Medicinal and aromatic plants as global resources. Acta Horticulturae **500**: 21-29.

HOLDSWORTH D (1986) Medicinal plants of Papua, New Guinea. In: Steiner RP (Ed.). Folk medicine: The Art and the Science. American Chemical Society, Washington DC.

HOSTETTMANN K (1986). Current level of technology in the separation, isolation and structural anlaysis of natural products. In: Barton D and Ollis WD (Eds.). Advances in Medicinal Phytochemistry. John Libbey and Company Ltd, London. Pp 67-76.

HOSETTMANN K and MARSTON A (1986). Plants used in African traditional medicine. In: Steiner RP (Ed.). Folk medicine: The Art and the Science. American Chemical Society, Washington DC.

HUSAIN A (1991). Economic aspects of exploitation of medicinal plants. In: Akerele O, Heywood V and Synge H (Eds.). The Conservation of Medicinal Plants. Proceedings of an International Consultation, 21st - 27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

HUSSEIN G, NAKAMURA N, MESELHY MR, HATTORI M (1999). Phenolics from *Maytenus senegalensis*. Phytochemistry **50**: 689-694.

HUTCHINGS A, SCOTT A, LEWIS A and CUNNINGHAM AB (1996). Zulu Medicinal Plants: An Inventory. University of Natal Press, Pietermaritzburg.

ICHIHARA Y, TAKEYA K, HITOTSUYANAGI Y, MORITA H, OKUYAMA S (1991). Cajucarinolide and isocajucarinolide: Anti-inflammatory diterpenes from *Croton cajucara*. Planta Medica **58**: 549-551.

ICHIMURA K and ODA M (1998). Stimulation of root growth of several vegetables by extracts from a commercial preparation of agar. Japan Society of Horticultural Science **67**: 341-346.

INGLIS TJJ (1997). Microbiology. 2nd Edition. Churchill Livingstone, Edinburg.

IWU WM (1994). African medicinal plants in the search for new drugs based on ethnomedical leads. In: Prance GT, Chadwick DJ and Marsh J (Eds.). Ethnobotany and the Search for New Drugs. Ciba Foundation Symposium 185. John Wiley and Sons, Chichester. Pp 116-129.

JÄGER AK, HUTCHINGS A, VAN STADEN J (1996). Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. Journal of Ethnopharmacology **52**: 95-100.

JÄGER AK and VAN STADEN J (2000). The need for cultivation of medicinal plants in southern Africa. Outlook on Agriculture **29**: 283-284.

JAIN N and BABBAR B (2002). Gum Katira – a cheap gelling agent for plant tissue culture medium. Plant Cell, Tissue and Organ Culture **71**: 223-229.

JAWORSKI EG (1978). Propagation of higher plants through tissue culture- A bridge between research and application. In: Hughes KW, Henke R and Constantin M (Eds.). Propagation of Higher Plants Through Tissue Culture - A Bridge Between Research and Application. Proceedings of an international Symposium held at the University of Tennessee, Knoxville. 16th - 19th April, 1978.

JOHNSON TS, NARAYAN SB and NARAYAMA DBA (1997). Rapid *in vitro* propagation of *Saussurea lappa*, an endangered medicinal plant, through multiple shoots cultures. *In Vitro* Cellular and Developmental Biology-Plant **33**: 128-130.

JONES OP (1991) The role of biotechnology in the multiplication and improvement of woody plants. Acta Horticulturae **289**: 35-44.

JORDAAN M and VAN WYK AE (1999). Systematic studies in subfamily Celastroideae (Celastraceae) in southern Africa: reinstatement of the genus *Gymnosporia*. South African Journal of Botany **65**: 177-181.

KAMO KK and MAHLEBERG PG (1988). Morphinan alkaloids: biosynthesis in plant (*Papaver* spp) tissue cultures. In: Bajaj YPS (Ed.). Biotechnology in Agriculture and Forestry. Vol. 4. Medicinal and Aromatic Plants. Springer-Verlag, Berlin. Pp 251-263.

KATAEVA NV, ALEXANDROVA IG, BUTENKO RG and DRAGAVTEEVA EV (1991). Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured *in vitro*. Plant Cell, Tissue and Organ Culture **27**: 149-154.

KATO A, MORIYASU M, ICHIMARU M, NISHIYAMA Y, JUMA FD, NG'ANG'A JN, MATHENGE SG, OGETO JO (1996) Isolation of alkaloidal constituents of *Zanthoxylum usambarense* and *Zanthoxylum chalybeum* using ion-pair HPLC. Journal of Natural Products **59**: 316-318.

KEVERS C, JACQUES P, THONART P and GASPAR T (1999). *In vitro* root cultures of *Panax ginseng* and *P. quinquefolium*. Plant Growth Regulation **27**: 173-178.

KIM YS, HAHN EJ, YEUNG EC and PAEK KY (2003). Lateral root development and saponin accumulation as affected by IBA or NAA in adventitious root cultures of *Panax ginseng* C.A. Meyer. *In Vitro* Cellular and Developmental Biology-Plant **39**: 245-249.

KINGHORN DA (1992). Plants as sources of medicinally and pharmaceutically important compounds. In: Nigg HN and Seigler D (Eds.). Phytochemical Resources for Medicine and Agriculture. Plenum Press, New York. Pp 75-95.

KOFI-TSEKPO WM (1997). Pharmaceutical applications of ethnobotany. In: Kinyua AM, Kofi-Tsekpo WM and Dangana L (Eds.). Conservation and Utilization of indigenous Medicinal Plants and Wild Relatives of Foods Crops. UNESCO, Nairobi Office. Pp 57-63.

KOHLEBACH HW and WERNICKE W (1977). Investigations on the inhibitory effect of agar and the function of active carbon in anther culture Z. Pflanzenphysiol. Bd **86**: 463-472.

KOKWARO JO (1976). Medicinal plants of east Africa. East African Literature Bureau, Nairobi.

KOKWARO JO (1987). Some common African herbal remedies for skin diseases with special reference to Kenya. In: Leeuwenberg AJM (compiler). Medicinal and poisonous plants of the tropics. Proceedings of symposium 5-35 of the 14th International Botanical Congress, Berlin, 25th July-1st August 1987.

KOKWARO JO (1991). Conservation of medicinal plants in Kenya. In: Akerele O, Heywood V and Synge H (Eds.). The Conservation of Medicinal Plants. Proceedings of an International Consultation, 21st -27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

KOKWARO JO (1993). Current status of utilization and conservation of medicinal plants in Africa south of Sahara. Acta Horticulturae **332**: 121-127.

KOKWARO JO (1995). Planta medica africana: Their role in health care. Whydah **4**: 1-7.

KOUMARE' M (1983). Traditional medicine and psychiatry in Africa. In: Bannerman RH, Burton, J and Wen-Chie C (Eds.). Traditional medicine and health care coverage. World Health Organization, Geneva.

KOWALSKI B and VAN STADEN J (1998). Formation of adventitious shoots directly on zygotic embryos of *Podocarpus henkelli.* South African Journal of Botany **64**: 368-369.

KUO YH, CHEN CH, KUO LMY, KING ML, WU TS, HARUNA M and LEE KH (1990). Antitumor agents, 112. [1]Emarginatine B, a novel potent cytotoxic sesquiterpene alkaloid from *Maytenus emarginata*. Journal of Natural products **53**: 422-428.

KUSAKARI K, YOKOYAMA M and INOMATA S (2000). Enhanced production of saikosaponins by root culture of *Bupleurum falcatum* L. using two-step control of sugar concentration. Plant Cell Reports **19**: 1115-1120.

KUTNEY JP, BEALE MH, SALISBURY PJ, STUART KL, WORTH BR, TOWNSLEY PM, CHALMERS WT, NILSSON K and JACOLI GG (1981). Isolation and characterization of natural products from plant tissue cultures of *M. buchananii.* Phytochemistry **20**: 653-657.

LANGE D (1997). Trade in plant material for medicinal and other purposes – a German case study. Traffic Bulletin **17**: 20-32.

LAUGHLIN CA and FAIRFIELD AS (1997). Natural products for the treatment of infectious diseases. In: Grifol F and Rosenthal J (Eds.). Biodiversity and Human Health. Island Press, Washington, DC. Pp 221-242.

LE GRAND A and WONDERGEM P (1989). Herbal Medicine and Health Promotion: A Comparative Study of Herbal Drugs in Primary Health Care. Royal Tropical Institute, Amsterdam.

LEAKEY R (1997). Domestication potential of *Prunus africana* in sub-saharan Africa. In: Kinyua AM, Kofi-Tsekpo WM and Dangana L (Eds.). Conservation and Utilization of indigenous Medicinal Plants and Wild Relatives of Foods Crops. UNESCO, Nairobi Office. Pp 99-106.

LEE N, WETZSTEIN HY and SOMMER HE (1986). The effects of agar vs. liquid medium on rooting in tissue-cultured Sweetgum. Horticultural Science **21**: 317-318.

LEITE JPV, RASTRELLI L, ROMUSSI G, OLIVEIRA AB, VILEGAS JHY, VILEGAS W and PIZZA C (2001). Isolation and HPLC quantitative analysis of flavonoid glycosides from Brazilian beverages (*Maytenus ilicifolia* and *M. aquifolium*). Journal of Agricultural and Food Chemistry **49**: 3796-3801.

LEWIS WH and ELVIN-LEWIS MPF (1977). Medical Botany: Plants Affecting Man's Health. John Wiley and Sons, New York.

LI JR and EATON GW (1984). Growth and rooting of Grape shoot apices *in vitro*. Horticultural Science **19**: 64-66.

LLOYD GB and McCOWN BH (1981). Commercially–feasible micropropagation of mountain laurel, *Kalmia latifolia*, by the use of shoot-tip culture. Combined Proceedings of the International Plant Propagation Society. **30**: 421-427.

LUI JHC and STABA EJ (1981). Effects of age and growth regulators on serially propagated *Digitalis lanata* leaf and root cultures. Planta Medica **41**: 90-95.

MABBERLEY DJ (1987) The Plant Book: A Portable Dictionary of the Higher Plants. Cambridge University Press.

MACKAY WA, TIPTON JL and THOMSON GA (1995). Micropropagation of Mexican redbud, *Cercis canadensis var. mexicana*. Plant Cell, Tissue and Organ Culture **45**: 295-299.

MALDONADO-MENDOZA IE, ALYORA-TALAVERA and LOYOLA-VARGAS VM (1992). Tropane alkaloid production in root cultures of *Datura stramonium*. *In vitro* Cell and Developmental Biology-Plant **28**: 62-72.

MAO AA, WETTEN A, FAY MF and CALIGARI PDS (2000). *In vitro* propagation of *Litsea cubeba* (LOURS.) Pers., a multipurpose tree. Plant Cell Reports **19**:263-267.

MARAIS W (1960). An enumeration of the *Maytenus* species of southern Africa. Bothalia **7**: 381-386.

MARÍN ML and MARÍN JA (1998). Excised rootstock roots cultured *in vitro*. Plant Cell Reports **18**: 350-355.

MARSHALL NT (1998). Searching for a Cure: Conservation of Medicinal Wildlife Resources in East and Southern Africa. TRAFFIC International, Cambridge.

MAUNDU PM, NGUGI GW, KABUYE CHS (1999). Traditional Food Plants of Kenya. National Museums of Kenya, Nairobi.

MAUNDU P, BERGER D, OLE SAITABAU C, NASIEKU J, KIPELIAN M, MATHENGE S, MORIMOTO Y and HÖFT R (2001). Ethnobotany of the Loita Maasai: towards community management of the forest of the lost child-experiences from the Loita ethnobotany project. People and Plants working paper No. 8. UNESCO, Paris.

McCHESNEY JD (1993). Biological and chemical diversity and the search for new pharmaceuticals and other bioactive natural products. In: Kinghorn AD and Balandrin MF (Eds.). Human Medicinal Agents from Plants. American Chemical Society, Washington DC. Pp 38-47.

McDONALD MS and WYNNE J (2003). Adventitious root formation in woody tissue: Peroxidase – a predictive marker of root induction in *Betula pendula*. *In vitro* Cellular and Developmental Biology-Plant **39**: 234-235.

McGAW LJ, JÄGER AK and VAN STADEN J (2002). Isolation of antibacterial fatty acids form *Schotia brachypetala*. Fitoterapia **73**: 431-433.

MEDEROS-MOLINA S (2002). *In vitro* propagation of *Maytenus canariensis* (Loes.) Kand. & Sund from apical meristem culture. Plant Tissue Culture **12**: 99-108.

MEYER HJ and VAN STADEN J (1991). Rapid *in vitro* propagation of *Aloe barbadensis* Mill. Plant Cell, Tissue and Organ Culture **26**: 167-171.

MONCOUSIN C (1991a). Rooting of microcuttings: general aspects. Acta Horticulturae **289**: 301-310.

MONCOUSIN C (1991b). Rooting of microcuttings: fundamental aspects. Acta Horticulturae **289**: 311-317.

MORRIS P, SCRRAGG AH, SMART NJ, STAFFORD A (1985). In: Dixon RA (Ed.). Plant cell Culture: A practical Approach. IRL Press, Oxford.

MUHAMMAD I, EL SAYED KA, MOSSA JS, AL-SAID MS, EL-FERALY FS, CLARK AM, HUFFORD CD, OH S and MAYER AMS (2000). Bioactive 12-oleanene triterpene and secotriterpene acids from *Maytenus undata* Journal of Natural Products **63**: 606-610.

MULLER-JAKIC B, MILLER M, PRÖBSTLE A, JOHNS TA, BAUER R (1993). Anti-inflammatory activity of *Zanthoxylum chalybeum* extracts and identification of protoberberines and benzophenanthridine alkaloids by GC-MS and HPLC. Planta Medica **59** (supplem), A664.

MULLINS MG, NAIR Y, SAMPET P (1979). Rejuvenation *in vitro*: induction of juvenile characters in adult clone of *Vitis vinifera* L. Annals of Botany **44**: 623-627.

MURASHIGE T and SKOOG F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15**: 473-497.

MURASHIGE T (1974). Plant propagation through tissue culture. Annual Review of Plant Physiology **25**:135-166.

MURASHIGE T (1977). Clonal crops through tissue culture. In: Barz W, Reinhard E and Zenk MH (Eds.). Plant Tissue Culture and its Biotechnological Application. Proceedings of the 1st International Congress on Medicinal Plants Research, Section B, held in Munich, Germany, September 6-10,1976. Springer-Verlag, Berlin. Pp 392-403.

MURASHIGE T and HUANG C (1987). Cloning plants by tissue culture: early years, current status and future prospects. Acta Horticulturae **21**: 35-42.

MURCH SJ, WIERENGA EJ, EL-DEMERDASH MA and SAXENA PK (2003). *In vitro* propagation of the Egyptian medicinal plant, *Echinops spinosissimus* Turra. Plant Cell, Tissue and organ Culture **74**: 81-86.

MURRAY PR, ROSENTAL KS, KOBAYASHI GS and PFALLER MA (1997). Medical Microbiology. 3rd Edition. Mosby, St Louis.

NETO EMC (1999). Traditional use and sale of animals as medicines in Feira de Santana city, Balia, Brazil. Indigenous Knowledge and Development Monitor **7**: 6-9.

NIGG HN AND SEIGLER D (1992). Future for natural products. In: Nigg HN and Seigler D (Eds.). Phytochemical Resources for Medicine and Agriculture. Plenum Press, New York. Pp 369-376.

NOZAKI H, MATSUURA Y, HIRONO S, KASAI R, CHANG JJ and LEE KH (1990). Antitumor agents, 116. Cytotoxic triterpenes from *Maytenus diversifolia*. Journal of Natural Products **53**: 1039-1041.

NYAMWAYA D (1986). Traditional health care in rural Kenya. The Courier **99**: 86-88.

NYAZEMA NZ (1987). Medicinal plants of wide use in Zimbabwe. In: Leeuwenberg, AJM (compiler). Medicinal and Poisonous Plants of the Tropics. Proceedings of symposium 5-35 of the 14th International Botanical Congress, Berlin, 25th July-1st August 1987.

ODERA J (1997). Traditional beliefs, sacred groves and home garden technologies: adapting old practices for conservation of medicinal plants. In: Kinyua AM, Kofi-Tsekpo WM and Dangana L (Eds.). Conservation and Utilization of Indigenous Medicinal Plants and Wild Relatives of Foods Crops. UNESCO, Nairobi Office. Pp 19-28.

OKWEMBA A. (2002). Tough rule for herbalists fixed. Daily Nation, 28th November.

ORABI KY, AL-QYASOUMI SI, EL-OLEMY MM, MOSSA JS AND MUHAMMAD I (2001). Dihydroagarofuran alkaloid and triterpenes from *Maytenus heterophylla and M. arbutifolia*. Phytochemistry **58**: 475-480.

PALEVITCH D (1991). Agronomy applied to medicinal plants conservation. In: Akerele O, Heywood V and Synge H (eds). The conservation of medicinal plants. Proceedings of an International Consultation, 21-27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

PAN MJ and VAN STADEN J (1998). The use of charcoal in *in vitro* culture – a review. Plant Growth Regulation **26**: 155-163.

PANAIA M, SENARATNA T, BUNN E, DIXON KW and SIVASITHAMPARAM K (2000). Micropropagation of the critically endangered western Australian species, *Symonanthus bancroftii* (F. Meull.) L. Haegi (Solanaceae). Plant Cell, Tissue and Organ Culture **63**: 23-29.

PANTANOWITZ D (1994). Alternative Medicine: A Doctor's Perspective. Southern Book Publishers (Pty) Ltd, Cape Town.

PATHAK U, PHUKAN S, KALITA MC and KALITA D (1998). *In vitro* root culture of *Boerhaavia diffusa* L. and study of the "punarnavoside" profile. Journal of Phytological Research **11**: 111-114.

PERDUE RE Jr, ABBOTT BJ and HARTNAL JL (1970). Screening plants for antitumour activity. II. Comparison of two methods of sampling herbaceous plants. Lloydia **33**: 1-6.

PEREIRA AMS, PEREIRA PS, MORAES FR, MORAES JRE and RODRIGUES DC (1993). Pharmacologically active compounds in plant tissue culture of *Maytenus ilicifolia* (CELASTRACEAE). Acta Horticulturae **333**: 205-209.

PEREIRA AMS, MORO JR, CERDEIRA RMM and FRANCA SC (1995). Effect of phytoregulators and physiological characteristics of the explants on micropropagation of *Maytenus ilicifolia*. Plant Cell, Tissue and Organ Culture **42**: 295-297.

PIERIK RLM (1987). *In Vitro* Culture of Higher Plants. Martimus Nijhoff Publishers, Dordrecht.

PLOTKIN MJ (1991). Traditional knowledge of medicinal plants - the search for new jungle medicines. In: Akerele O, Heywood V and Synge H (Eds.). The Conservation of Medicinal Plants. Proceedings of an International Consultation, 21st - 27 March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

POOLEY E (1993). The Complete Field Guide to Trees of Natal, Zululand and Transkei. Natal Flora Publications, Durban.

PRANCE GT (1994). Intoduction. In: Prance GT, Chadwick DJ and Marsh J (Eds.) Ethnobotany and the Search for New Drugs. Ciba Foundation Symposium 185. John Wiley and Sons, Chichester.

PRINCIPE PP (1991). Valuing the biodiversity of medicinal plants. In: Akerele O, Heywood V and Synge H (Eds.). The Conservation of Medicinal Plants. Proceedings of an International Consultation, 21st -27th March 1988 held at Chiang Mai, Thailand. Cambridge University Press.

PUSHPANGADAN P and THOMAS J (1999). A novel experiment in the *ex situ* conservation of medicinal and aromatic plants genetic diversity. Acta Horticulturae **500**:177-182.

RABE T and VAN STADEN J (1997). Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethnopharmacology **56**: 81-87.

RABE T and VAN STADEN J (1998). Screening of *Plectranthus* species for antibacterial activity. South African Journal of Botany **64**: 62-65.

RABE T and VAN STADEN J (2000). Islolation of an antibacterial sesquiterpenoid from *Warbugia salutaris*. Journal of Ethnopharmacology **73**: 171-174.

RASOANAIVO P and RATSIMAMANGA-URVEG S (1993). Biological evaluation of plants with reference to the Malagasy flora. Monograph of the IFS-NAPRECA workshop on bioassays, Antananarivo, Madagascar.

RATHORE TS, DEORA NS and SHEHAWAT NS (1992). Cloning of *Maytenus emarginata* (Willd.) Ding Hou-a tree of the Indian desert, through tissue culture. Plant Cell Reports **11**: 449-451.

ROBBINS C (1997). United States of America plant trade studies. Traffic Bulletin **16**: 121-125.

ROBERTS MF (1988). Medicinal products through plant biotechnology. In: Robins RJ and Rhodes MJC (Eds.). Manipulating Secondary Metabolism in Culture. Cambridge University Press, Cambridge. Pp 201-216.

ROBSON NKB, HALLE N, MATHEW N and BLAKELOCK R (1994). Celastraceae. In: Pollhill RM (Ed.). Flora of Tropical East Africa. Balkema, Rotterdam.

ROGERS RB and SMITH MAL (1992). Consequences of *in vitro* and *ex vitro* root initiation for miniature rose production. Journal of Horticultural Science **67**: 535-540.

ROGERS CB, ABBOTT ATD, VAN WYK AE (2000). A convenient thin layer chromatographic technique for chemotaxonomic application in *Maytenus* (Celastraceae). South African Journal of Botany **66**: 7-9.

ROODT V (1998). Trees and Shrubs of the Okavango Delta: Medicinal Uses and Nutritional Value. Shell Oil, Botswana (Pty) Ltd, Gaborone.

RUSSEL AD and CHOPRA I (1996). Understanding Antibacterial Action and Resistance. 2nd Edition Ellis Horwood, London.

SANCHEZ MC, SAN-JOSE MC, BALLESTER A and VIEITEZ AM (1996). Requirements for *in vitro* rooting of *Quercus robur* and *Q. rubra* shoots derived from mature trees. Tree Physiology **16**: 673-680.

SANDAL I, BHATTACHARYA A, AHUJA PS (2001). An efficient liquid culture system for tea shoot proliferation. Plant Cell, Tissue and Organ Culture **65**:75-80.

SCHRIPSEMA J, FUNG SY and VERPOORTE R (1996). Screening plant cell cultures for new industrially interesting compounds. In: Dicosmo F and Misawa M (Eds.). Plant Cell Culture Secondary Metabolism. CRC Press, LLC.

SEABROOK JEA (1980). Laboratory Culture. In: Staba EJ (Ed.). Plant Tissue Culture as a Source of Biochemicals, CRC Press, Inc, NY.

SEKAR KVS, CAMPAGNE JM and SNEDEN AT (1996). 5-Benzoyl-5-deacetylwilforidine: a new sesquiterpene nicotinoyl alkaloid from *Maytenus buchananii*. Planta Medica **62**: 368-370.

SHALE TL, STIRK WA and VAN STADEN J (1999). Screening of medicinal plants used in Lesotho for antibacterial and anti-inflammatory activity. Journal of Ethnopharmacology **67**: 347-354.

SHANKS JV and MORGAN J (1999). Plant 'hairy root' cultures. Current Opinion in Biotechnology **10**: 151-155.

SHARMA AK, PRASAD RN and CHATURVEDI HC (1981). Clonal propgatioin of *Bougainvillea glabra* "Magnifica" through shoot apex culture. Plant Cell, Tissue Organ Culture **1**: 33-38.

SHARP WR and LARSEN PD (1979). Plant cell and tissue culture: current applications and potential. In: Sharp WR, Larsen PD, Paddock E and Raghavan V (Eds.). Plant Cell and Tissue Culture: Principles and Applications. Ohio State University Press, Columbus. Pp 115-120.

SHELDON JW, BALICK MJ and LAIRD SA (1997). Medicinal plants: can utilization and conservation coexist? The New York Botanical Gardens, Bronx, NY, USA.

SHIMOMURA K and KITAZAWA T (1991). Tanshinone production in adventitious roots and regenerates of *Salvia miltiorrhiza*. Journal of Natural Products **54**: 1583-1587.

SHORT KC, WARBURTON J and ROBERTS AV (1987). *In vitro* hardening of cultured Cauliflower and *Chrysanthemum* plantlets to humidity. Acta Horticulturae **212**: 329-334.

SHROENTAL R (1972). Herbal medicines to avoid. Nature **238**: 106-107.

SINDIGA I (1994). Indigenous (medical) knowledge of the Maasai. Indigenous Knowledge and Development Monitor **2**: 16-18.

SINDIGA I (1995). African ethnomedicine and other medical systems. In: Sindiga I, Nyaigototti-Chacha C and Kanuna MP (Eds.).Traditional Medicine in Africa. East African Educational Publishers Ltd, Nairobi. Pp 16-29.

SINGH S, RAY BK, MATHEWS S, BURAGOHAIN P, GOGOI J, SHARMA BK, and DEKA PC (1999). Micropropagation of a few medicinal plants. Annals of Biology-Ludhiana **15**: 1-7.

SINHA RK, MAJUMDAR K and SINHA S (2000). *In vitro* diffrentiation and plant regeneration of *Albizia chinenesis* (Osb.) Merr. *In vitro* Cellular and Developmental Biology-Plant **36**: 370-373.

SLEIGH JD and TIMBURY MC (1998). Note on Medicinal Bacteriology. 5th Edition. Churchill Livingstone, New York.

SOFOWORA A (1982). Medicinal Plants and Traditional Medicine in Africa. John Wiley and Sons, Chichester.

SOMMER HE and CALDAS LS (1981). *In vitro* methods applied to forest trees. In Thorpe TA (Ed.). Plant Tissue Culture: Methods and Applications in Agriculture, Academic Press, Iinc, NY

SREEKUMAR S, SEENI S and PUSHPANGADAN P (2000). Micropropagation of *Hemidesmus indicus* for cultivation and production of 2-hydroxy-4-methoxy benzaladehyde. Plant Cell, Tissue and Organ Culture **62**: 211-218.

SRIKANDARAJAH S and MULLINS MG (1981). Micropropagation of Granny Smith apple: factors affecting root formation in vitro. Journal of Horticultural Science **56**: 71-76.

SUFFNESS M and DOUROS J (1982). Current status of the NCI plant and animal product program. Journal of Natural Products **45**: 1-15.

SUMITA J, SAHU NP, JAYANTI S, JHA TB, MAHATO SB, JHA S and SEN J (1991). Production of emetin and cephaeline from cell suspension and excised root cultures of *Cephaelis ipecacuanha*. Phytochemistry **30**: 3999-4003.

TANIGUCHI M and KUBO I (1993). Ethnobotanical drug discovery based on medicinemen's trials in the African savanna: Screening of East African plants for antimicrobial activity II. Journal of Natural Products **56**: 1539-1546.

TANIGUCHI S, YAZAKI K, YABU-UCHI R, KAWAKAMI K, ITO H, HATANO T and YOSHIDA T (2000). Galloylglucoses and riccionidin A in *Rhus javanica* adventitious root cultures. Phytochemistry **53**: 357-363.

TAYLOR JLS and VAN STADEN J (2001). The effect of nitrogen and sucrose concentrations on the growth of *Eucoms autumnalis* (Mill.) Chitt. plantlets *in vitro*, and on subsequent anti-inflammtory activity in extracts prepared from the plantlets. Plant Growth Regulation **34:** 49-56.

TAYLOR JLS, RABE T, McGAW LJ, JÄGER AK and VAN STADEN J (2001). Towards the scientific validation of traditional medicinal plants. Plant Growth Regulation **34**: 23-37.

TESHA AJ (1991). Effects of rise in population on the endangered plant species in Tanzania. A paper presented during the fourth NAPRECA symposium on natural products. December 16th –21st, Addis Ababa, Ethiopia.

THORPE TA (1990). The current status of plant tissue culture. In: Bhojwani SS (Ed.). Plant Tissue Culture: Applications and Limitations. Elsevier Science Publishing Company Inc.. New York.

TOWERS NGH and ELLIS S (1993). Secondary metabolism in plant tissue cultures transformed with *Agrobacterium tumefaciens* and *A. rhizogenes*. In: Kinghorn AD and Balandrin MF (Eds.). Human Medicinal Agents from Plants. American Chemical Society, Washington DC. Pp 56-78.

TYLER VE (1986). Plant drugs in the 21st century. Economic Botany **40**: 279-288.

ULRICH JM (1962). Cultural requirements for growth of excised *Ponderosa* pine roots. Physiologia Plantarum **15**: 59-71.

VAN STADEN J (1999). Medicinal plants in southern Africa: utilization, sustainability, conservation-can we change the mindsets? Outlook on Agriculture **28**: 75-76.

VAN UDEN W, HOMAN B, WOERDENBAG HJ, PRAS N, MALINGRE TM, WICHERS H J, HARKES M and VAN UDEN W (1992). Isolation, purification and cytotoxicity of 5-methoxypodophyllotoxin, a lignan from a root culture of *Linum flavum*. Journal of Natural Products **55** 102-110.

VAN WAES J (1987). Effect of activated charcoal on *in vitro* propagation of western European Orchids. Acta Horticulturae **212**: 131-138.

VAN WYK B, VAN WYK P and VAN WYK BE (2000). Photographic Guide to Trees of Southern Africa. Briza Publications, Pretoria.

VANE J (1994). Towards a better aspirin. Nature **367**:215-216.

VANE JR and BOTTING RM (1996). Mechanism of action of anti-inflammatory drugs. Scandanavian Journal of Rheumatology **(Suppl.102)**: 9-21.

VÁZQUEZ-FLOTA FA and LOYOLA-VARGAS VM (2003). *In vitro* plant cell culture as the basis for the development of a research institute in Mexico: Centro De Investigción Científica de Yucatán. *In Vitro* Cellular and Developmental Biology-Plant **39**: 250-258.

VLIENTINCK AJ, VAN HOOF L, TOTTÉ J, LASURE A, VANDEN BERGHE D, RWANGABO PC and MWAKIYUMWANI J (1995). Screening of a hundred Rwadese medicinal plants for antibacterial and antiviral properties. Journal of Ethnopharmacolology **46**: 31-47.

WAINWRIGHT H and ENGLAND N (1987). Micropropagation of *Prosopis juliflora* (Swartz) D.C.: establishment *in vitro*. Acta Horticulturae **212**: 49-54.

WATT JM and BREYER-BRANDWIJK MG (1962). The Medicinal and Poisonous Plants of Southern and Eastern Africa. 2nd Edition. Livingstone, London.

WEEKS JR (1973). The prostaglandins: Their nature, formation and general pharmacology. In: Kahn RH and Lands WE (Eds.). Prostaglandins and Cyclic AMP: Biological Actions and Clinical Applications. Academic Press, New York.

WHITE HL and GLASSMAN AT (1974). A simple radiochemical assay for prostaglandin synthetase. Prostaglandins **7**: 123-129.

WHO (2002). WHO Traditional Medicine Strategy 2002-2005. (http://www.who.int/nedicines/organization/trm/orgtrmdef.shtml). Accessed on 6th April, 2003.

WIELGORSKAYA T (1995). Dictionary of Generic Names of Seed Plants. Columbia University Press. New York.

WILHELM E (2000). Somatic embryogenesis in oak (*Quercus* spp). *In Vitro* Cellular and Developmental Biology-Plant **36**: 349-357.

WILKINS CP and DODDS JH (1983). Tissue culture conservation of woody species. In: Dodds JH (Ed.). Tissue Culture of Trees. Kroom Helm Ltd, Beckenham.

WILLIAMS RR, TAJI AM and BOLTON JA (1985). Specificity and interaction among auxins, light and pH in rooting of Australian woody species *in vitro*. Horticultural Science **20**: 1052-1053.

WOCHOK ZS (1981). The role of tissue culture in preserving threatened and endangered plant species. Biological Conservation **20**: 83-89.

WOO SH, PARK JM and YANG JW (1997). Induction of branch roots by cutting method in *Hyoscamus niger* root culture. Plant Cell, Tissue and Organ Culture **48**: 131-134.

WYNNE J and McDONALD MS (2002). Adventitious root formation in woody plant tissue: The influence of light and indole-3-butyric acid (IBA) on adventitious root induction in *Betula pendula*. *In Vitro* Cellular and Developmental Biology-Plant **38**: 210-212.

YAM TW, ERNEST R, ARDITTI J, NAIR H and WEATHERHEAD MA (1990). Charcoal in orchid seed germination and tissue culture media: A review. Lindleyana: The Scientific Journal of the Orchid Society **5**: 256-265.

YAMANDA Y and HASHIMOTO T (1988). Biosynthesis of tropane alkaloids. In: Bock G and Marsh J (Eds.). Application of Plant Cell and Tissue Culture. Ciba Foundation Symposium 137. John Wiley and Sons, Chichester. Pp 199-212.

YONDER PS (1982). Issues in the study of ethnomedical systems in Africa. In: Yonder PS (Ed.). African Health and Healing Systems. Crossroads Press, Los Angeles.

ZHANG X (1996). Traditional medicine and WHO. World Health (WHO Magazine) **2**: 4-5.

ZIMMERMAN RH (1983). Factors affecting *in vitro* propagation of apple cultivars. Acta Horticulturae **131**: 171-178.

ZSCHOCKE S, RABE, TAYLOR JLS, JÄGER AK and VAN STADEN J (2000). Plant part substitution-a way to conserve endangered medicinal plants? Journal of Ethnopharmacology **71**: 281-292.