MEDICINAL PROPERTIES AND MICROPROPAGATION OF *CUSSONIA* SPECIES

by

Pokazi Tetyana

Submitted in fulfilment of the requirements
for the degree of
Master of Science
December 2000

in the

School of Botany and Zoology
Faculty of Science and Agriculture
University of Natal, Pietermaritzburg

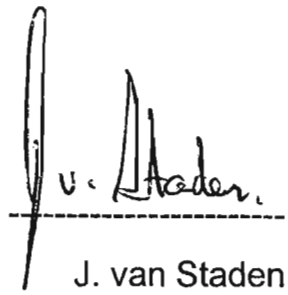
PREFACE

The experimental work described was carried out in the Botany Department, University of Natal, from January 1999 to December 2000; under the supervision of Professor J. van Staden.

This work has not been submitted in any form to another university and, except where the work of others is acknowledged in the text, is the result of my own investigation.

P. Tetyana

I declare that the above statement is correct.



J. van Staden
(Supervisor)

PUBLICATIONS FROM THIS THESIS

TETYANA P. and VAN STADEN J. (2000) Micropropagation of *Cussonia paniculata*, a medicinal plant with horticultural potential. *South African Journal of Botany* (In Press).

CONFERENCE CONTRIBUTIONS FROM THIS THESIS

1. **TETYANA P. and VAN STADEN J.** (2000) Micropropagation of *Cussonia paniculata* (Paper). **Twenty-sixth Annual Congress of South African Association of Botanists, University of Potchefstroom, Potchefstroom.**

2. **TETYANA P., MEYER J. J. M., PROZESKY E. A., JÄGER A. K. and VAN STADEN J** (2000) Screening of *Cussonia* species for medicinal properties (Poster). **Twenty-sixth Annual Congress of South African Association of Botanists, University of Portchefstroom, Portchefstroom.**

ACKNOWLEDGEMENTS

My sincere thanks are due to the many individuals who have contributed towards this project:

My supervisor, Professor J. van Staden, for his guidance, enthusiasm and encouragement during the course of my project.

The members of my research committee, Dr A. K. Jäger and Miss C. W. Fennell for their helpful advice throughout the project.

The National Research Fund and Melon Foundation for financial assistance.

Professor J. J. M. Meyer and Dr E. A. Prozesky for helping with the anti-malarial work in their laboratory at the University of Pretoria.

The Staff and Colleagues in the Research Centre for Plant Growth and Development for their effective assistance and unfailing patience.

Mr and Mrs V. H. R. Ford for collection of plant material from their farm.

My father, Mzukisi Mabhelonke and mother, Eunice Zolisa Maccelina Tetyana; and the rest of the family for their love and support.

My dear friends, especially Lumka Majola and Nomakwezi Mzilikazi for supporting and encouraging me during good and bad times.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA	-	Abscisic acid
BA	-	Benzyladenine
2,4-D	-	2,4-Dichlorophenoxyacetic acid
GA_3	-	Gibberellic acid
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyric acid
iP	-	Isopentenyladenine
MS	-	Murashige and Skoog medium (1962)
NAA	-	∝-Naphthaleneacetic acid

ABSTRACT

Cussonia species (commonly known as Cabbage trees) are indigenous to South Africa and are used in traditional medicine to treat an assortment of diseases. Due to their attractive growth form, they are assets in gardens. However, there are no developed methods for propagating these species. The use of three selected species, *Cussonia paniculata* (Eckl. & Zeyh.), *C. spicata* (Thunb.) and *Schefflera umbellifera* (Sond.) Baill. (= *C. umbellifera*), in traditional medicine was validated. Rapid propagation protocols for *C. paniculata* and *C. spicata* were investigated and ultimately developed for the former species.

Cussonia paniculata, *C. spicata* and *C. umbellifera* were screened for their medicinal properties, mainly focussing on anti-bacterial, anti-inflammatory and anti-malarial activities. In the anti-bacterial screening, *C. spicata* bark and root extracts showed activity against selected Gram-positive and Gram-negative bacterial strains at a concentration of 50 mg ml^{-1}. The highest inhibition was observed with ethanol and ethyl acetate root extracts against *Staphylococcus aureus*. The other two species did not show anti-bacterial activity. Ethanol and ethyl acetate extracts of all species showed anti-inflammatory activity in the cyclooxygenase assay (COX-1) at a concentration of 8 µg ml^{-1}. These active extracts showed an inhibition percentage that was greater than 50 % against cyclooxygenase. In the anti-malarial screening, bark extracts were screened. *C. umbellifera* bark extracts exhibited the best inhibition against *P. falciparum*, a malaria-causing agent in humans. The percentage inhibition of these extracts was up to 100% at a concentration of 200 µg ml^{-1}. While *C. spicata* is known to be used to treat malaria, the screening results showed much less activity (less than or equal to 35 %) as compared to *C. umbellifera*, which is preferably used to treat malaria. The results obtained from screening these three species

validated their use in traditional medicine. This means that the people or traditional healers use these species for different treatments by possibly relying on past knowledge about the effects after administering the medicine.

Fingerprinting using Thin Layer Chromatography (TLC) was used in an attempt to determine whether there are any chemical differences or similarities between the three species. There were similarities between the plant parts across the species as well as some differences. However, this method cannot be used as an unequivocal test to deduce that compounds that are present in a certain species and not in others are the ones responsible for bringing about a certain biological activity. That can only be achieved by a bioassay-guided isolation of possible compounds.

A tissue culture protocol was developed to produce a large number of plants of *C. paniculata*. Explants were derived from nodal explants of *in vitro* germinated seeds and cultured on Murashige and Skoog (MS) (1962) medium supplemented with 3% sucrose, 2.5 mg l^{-1} BA and solidified with 3 g l^{-1} Gelrite. These explants produced multiple shoots. The average number of shoots per explant ranged between 1 to 3.5. Multishoots were subcultured on to rooting media and roots were produced on MS with 0.75 mg l^{-1} IBA and 1 mg l^{-1} NAA. Callus from zygotic embryos also produced plantlets on MS supplemented with 1.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA. Hyperhydricity was encountered in this study. This problem was reversed successfully by transferring the shoots from medium solidified with 3 g l^{-1} Gelrite to medium solidified with 8 g l^{-1} agar. Plantlets were successfully acclimatized for planting *ex vitro*. The percentage of healthy plants after a 35-day acclimatization period was 63 %.

C. spicata was not successfully micropropagated from shoot-tip explants. However, a protocol was developed for decontaminating shoot-tips from the

mother plants. The plant material was successfully decontaminated with 0.01% $HgCl_2$ for 15 min. The decontamination percentage was up to 80 %. Browning of the explants was observed and it was successfully treated with soaking the explants in a 15 mg l^{-1} ascorbic acid solution for 15 min. A high percentage of shoot-tip regeneration (80 %) was observed when they were cultured on MS medium supplemented with 2 mg l^{-1} BA, 1 mg l^{-1} IAA and 1 mg l^{-1} GA_3. However, multishoots were not observed as in *C. panicualata*. Shoot elongation *in vitro* was similar to shoot elongation as it occurs in nature. The shoots elongated and a flush of palmitately arranged leaves were produced. Further research is required to investigate a commercially viable protocol for rapid propagation and conservation of the germplasm of *Cussonia* species.

CHAPTER 1

LITERATURE REVIEW

1.1 Ethnobotany and traditional medicine

The natural world once provided all the medicinal agents used by man, with higher plants constituting the principal source (BALANDRIN, KINGHORN and FARNSWORTH 1993). BALANDRIN, KINGHORN and FARNSWORTH (1993) further estimated that approximately 80% of the developing world rely on traditional medicine. Eighty five percent of traditional medicines are derived from plants and their extracts. Therefore, it can be extrapolated that 3.5 billion people rely on plants for medicine. Presently, natural compounds, their derivatives and analogues still represent over 50% of all drugs in clinical use, with higher plant-derived natural products representing 25% of the total (BALANDRIN KINGHORN and FARNSWORTH 1993). Plant-derived products therefore represent stable markets upon which both physicians and patients rely.

Primarily, traditional healers would harvest what they need, prepare plant material in many different ways and administer medicines at their discretion. Scientific investigation of medicinal plants to obtain specific knowledge about their medicinal properties and to isolate active compounds responsible for healing is a relatively recent event. Some important plant-derived drugs and intermediates are still obtained commercially by extraction from whole plants. Traditional cultures still rely, largely, on wild medicinal plants supplemented by cultivation in home

gardens, scaled to the need of an extended family.

Because of the need for a large variety of medicines, market demands are often spread over diverse terrains and microclimates. Therefore, in the past they have rarely generated enough pressure to seriously threaten a resource. However, rapid population growth and the resulting increased demand are overwhelming this traditional balance. Relatively few plants have been surveyed systematically for biologically active chemical constituents (BALANDRIN, KINGHORN and FARNSWORTH 1993). Thus, it may be expected that new plant sources of valuable and pharmaceutically interesting materials remain to be discovered and developed.

The use of ethnobotanical data can provide a short cut in pharmacological research by indicating plants with specific folk-medicinal uses which might be likely sources of biological active chemicals (GENTRY 1993). Unfortunately, knowledge of medicinal uses of plants is a thinly spread residue of folk medicine, weakened by colonialism or other fragmentation. Traditional medicine is based on long-term treatment of chronic problems rather than their symptoms. Unlike modern commercial practice, it is not easy to replicate, and the dosages administered are not easy to quantify. Thus, its use necessarily involves not only trust in the healer, but also belief in the ability of the medicine to cure and the capability of the medicine man to administer the right dosage (WAANE 1990).

In southern Africa, indigenous African medicine coexists with western allopathic medicine, western herbalism, homeopathy, Ayurvedic medicine

from India and Traditional Chinese medicine. It is possible that unifying therapeutic principles will ultimately be found, probably based on subtle energies acting along the body-mind continuum that will unite healing systems which at present appear to be quite distinct from each other (VAN WYK and GERICKE 2000). However, with reference to South Africa, traditional healers have not officially been recognised by State authorities. The situation is now changing and there has for some time been active movement towards the integration of traditional healing into the official health care system (GUMEDE 1989, PICK 1992). Through programmes, in collaboration with traditional healers, traditional medicine is now being integrated into western medicine, with scientific research playing a big role in this transition (WAANE 1990).

The World Health Organisation encourages the incorporation of useful elements of traditional medicine into national health care systems (AKERELE 1991). This requires an inventory of periodically updated therapeutic classification of medicinal plants. Methods of safe and effective use of medicinal plant products have to be carefully outlined. A periodic check on scientific criteria and methods for assessing the safety of medicinal plant products and their efficacy in the treatment of specific conditions and diseases is also required.

Traditional healers today tend to pose a threat to the survival of the plants that they use. Often, the plant material is harvested destructively so that the plant cannot regenerate and survive. Sometimes the whole plant is harvested, especially if the part of the plant sought-after is underground. This is turn destroys the environment and does not ensure a sustainable

resource. Nowadays, traditional healers and their subjects are being taught methods of non-destructive harvesting by nature conservationists and nurseries. These methods ensure a long-term sustainable resource and a standard conservation status of plants. Most of these methods are conventional methods of propagation including the use of cuttings and raising plants from seeds. Conventional methods of propagation can be slow as they are also influenced by seasonal changes and therefore do not always ensure readily available plants.

Nevertheless, there are new biotechnological methods that can be employed to ensure availability of plants. Plant tissue culture is a technology by which clones of the same plant can be produced on a large scale. This technology involves propagation of disease-free plants under controlled environments. Tissue culture methods have greatly increased the potential of propagation by exploiting regenerative behaviour more efficiently and in a wider range of plants than is possible with conventional procedures (HUSSEY 1980). This method is an alternative to conventional methods because it is fast and has been successfully employed to obtain propagules from a wide range of plant species especially those frequently used. Apart from unlimited, all-year-round supply of plants to traditional healers, this method provides a guaranteed method of conservation. It may also ensure that species used medicinally are never depleted and therefore will not become threatened. Cloning by means of tissue culture is an alternative to the traditional methods of plant propagation, and could ensure a higher quality product (BONGA 1982). Ultimately plants with the highest level of active components will be the most profitable to propagate.

1.1.1 Role of *Cussonia* species in traditional medicine

Species of the genus *Cussonia* are used in traditional medicine against pain, inflammation and infection (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). The macerated roots of *Cussonia spicata* are used by the Zulu to treat malaria (COATES PALGRAVE, COATES PALGRAVE, and COATES PALGRAVE 1985). In Nigeria, *C. spicata* is used for treatment of mental disease, malaria, rheumatism and dysmenorrhoea (DUBOIS, ILYAS and WAGNER 1986). *C. natalensis* found in KwaZulu-Natal and Gauteng Province contains an active medicinal compound, oleanoic acid (a pentacyclic triterpene acid), with anti-ulcer properties (FOURIE, MATTEE and SNYCKERS 1989). The compound was extracted from the twigs and leaves.

The Sotho people administer a decoction of the leaves as a treatment of early nervous and mental diseases. The southern Sotho use the plant as a remedy for colic and dysmenorrhoea. Nyamwezi (Zimbabwe), Zulu and northern Sotho use the bark of the *Cussonia sp.* as a gonorrhoea remedy (WATT and BREYER-BRANDWIJK 1962). Along the eastern border of southern Zimbabwe, the bark is also used as a malaria remedy (WATT and BREYER-BRANDWIJK 1962). In South Africa, the leaves of *Cussonia sp.* are generally used to treat rheumatism, colic and insanity.

1.1.1.1 Pharmacological effects

Most of the saponins of official saponin drugs are triterpene glycosides. Sugar residues may be linked via the OH group at C-3-OH of the aglycone

(monodesmosidic) or more rarely via two OH groups or a single OH group and a carboxyl group of the aglycone moiety (bisdesmosidic saponins). Most triterpene saponins possess haemolytic activity, which varies from strong to weak depending on the substitution pattern. Steroid glycosides specifically affect the dynamics of the heart muscle (WAGNER and BLADT 1996). Three major classes of saponins, triterpene glycosides, steroid glycosides and the glycoalkaloids are found in *C. spicata.* Cussonoside A and B are triterpene saponins isolated from *Cussonia barteri.* Cussonoside A is known to have a sedative effect (DUBOIS, ILYAS and WAGNER 1986).

Molluscicidal saponins, among others, are plant-derived compounds that have a biological activity against amphibious snails. *C. spicata* contains molluscicidal saponins, which can be isolated from the stem bark (GUNZINGER, HOSTETTMANN and MSONTHI 1986).

Polyacetylenes are common constituents of the Araliaceae, and are of interest to plant physiologists and pharmacologists because of their anti-bacterial, anti-fungal, and other biological activities. A bioassay-guided isolation of a C18-polyacetylene from *C. barteri* has been performed recently by PAPAJEWSKI, GUSE, KLAIBER, ROOS, SÜßMUTH, VOGLER, WALTER and KRAUS (1998). This compound has anti-bacterial action against *Bacillus subtilis* and *Pseudomonas fluorescens*, an anti-fungal action against *Cladosporium cucumerium,* molluscicidal action against *Biomphalaria glabrata* at low concentrations, as well as haemolytic action.

1.1.1.2 Other uses

Cussonia paniculata trees are regarded as fodder trees, cattle relish them. The plant has been suspected of causing poisoning in cattle but an oral administration of 200 g to a sheep failed to produce any symptoms (WATT and BREYER-BRANDWIJK 1962). Although the wood is soft, it is tough and fibrous and has been used for making brake blocks. The Xhosa use the raw fruit of a *Cussonia* species as an article of diet. The trees are also good for providing shade.

The Lobedu (Sotho) wash the new born with an infusion of the powdered root of *Cussonia spicata* daily until the infant leaves the hut for the first time. This is usually after five to seven days but the washing may extend over one and a half months. The idea underlying this application is to prevent skin irritation and pimples (zwiso) and to make the infant strong and fat. The Lobedu also add the flower to tobacco in making snuff with the idea of giving it a "proper taste" (WATT and BREYER-BRANDWIJK 1962).

1.1.2 Horticultural potential of *Cussonia* species

Cussonia trees have a beautiful growth form and attractive foliage. The leaves have distinct pale bluish and grey-green colours (COATES PALGRAVE, COATES PALGRAVE and COATES PALGRAVE 1985). *C. spicata* leaves have a shiny green colour. These trees have a potential for horticultural use and are becoming very popular for decorative purposes in gardens. In a study made by WALKER (1988), he discovered the potentiality of *C. paniculata* to be used as foliage pot plants. This species is

listed among trees that are considered decorative for small gardens. The trees of *C. paniculata* species are usually short (about 3 m) with unique-shaped umbrella foliage.

Cabbage trees are great assets in the garden as they take up very little space. The combination of a thin unbranched trunk surmounted by a mop of leaves is unique. The unusual growth form makes good specimens and adds new texture to garden structure. The trees can also be utilized for landscape work (COATES PALGRAVE, COATES PALGRAVE and COATES PALGRAVE 1985). There are *Cussonia* species that have variegated leaves. If a method of propagating this genus could be established, particular "sports" can be propagated for ornamental purposes.

1.1.3 Conservation status and propagation

Some trees and shrubs are in danger of being "lost" through the depletion of indigenous forests. This is caused by the encroachment of agriculture and towns, drought and fires. The traditional healers also play a role in the depletion by harvesting whole plants or parts of plants for medicinal use. One method of conserving is to cultivate successfully. *Cussonia* trees are usually grown from seeds. It takes between 15-30 days for the seeds to germinate in a suitable environment. The trees increase in size around 80 cm to 1 m per year (VENTER and VENTER 1996).

Cussonia species are indigenous, and are protected in all South African provinces (PALMER and PITMAN 1961, JOHNSON and JOHNSON 1993).

Cussonia was selected as the tree of the year in 1987 by the Forestry branch of the Department of Environmental Affairs in South Africa.

There are no known methods, other than by seed, to propagate *Cussonia* trees. Therefore, an opportunity arises to explore other methods such as tissue culture for rapid propagation.

1.1.4 Morphology and distribution of *Cussonia* species

The genus *Cussonia* (Thunb.) belongs to the Araliaceae and is composed of 18 or more species. This genus was discovered by, and named after PIERRE CUSSONIN (1727-1783), Professor of Botany, University of Montpellier (PALMER and PITMAN 1961, JOHNSON and JOHNSON 1993). The trees of this genus are commonly known as Cabbage trees, with traditional names used by different cultural groups. The species which are indigenous and well distributed all over the eastern and southern part of South Africa are *C. spicata, C. natalensis, C. paniculata, C. arenicola, C. sphaerocephala, C. nicholsonii* (STREY 1973).

C. natalensis is the most attractive of all Cabbage trees and grows in the eastern half of KwaZulu-Natal and the Eastern and Northern Free State, where there are rocky slopes. *Cussonia paniculata* (Eckl. & Zeyh.) belongs to the subgenus *Paniculata*. This subgenus is monotypic. It may be distinguished from all the other species by the paniculate branches of the inflorescence and the elongate simple, shallowly to deeply lobed leaflets, which are never vertebrate. *C. paniculata* can be found in rocky, high altitude grassland and in small bush clumps. This species is mostly

abundant around Johannesburg and Pretoria. *Cussonia umbellifera* (Sond.) is a species of which the name was changed. It was placed under *Schefflera*. Its name was changed to *Schefflera umbellifera* (Sond.) Baill. This species is closely related to the *Cussonia* species (STREY 1973).

When all the South African species of *Cussonia* are considered, it is soon evident that they do not form a homogenous assemblage of species, but may be divided into several groups of closely related species (STREY 1973). However, *Cussonia spicata* (Thunb.) (Figure 1A-D) and *C. sphaerocephala* (Strey.) represent the typical *Cussonia* (STREY 1973). *C. spicata* is well distributed in KwaZulu-Natal and the Eastern Cape region and is common in cool grasslands, highveld and dry woodlands.

The trees are evergreen and can grow up to 10 m tall, with an erect growth. Wood is soft, light, very coarse and fibrous and the roots are tuberous. The leaves resemble no other species. They are palmately compound, radiating from the central point and are arranged in whorls. In shape, all the leaves vary from simple and deeply lobed to singly and doubly compound.

The flowers are yellowish to greenish in colour. Not all the ovaries are fertilized during fertilization. Fertile ovaries increase in size and develop a fleshy exocarp attractive to birds. Each fruit contains one or two seeds (Figure 1E). The seeds are less than 5 mm in length and have a seed coat. Seeds of *C. spicata* grow more easily in the field than the other species (VENTER and VENTER 1996). In all species of *Cussonia* the structures that bear fruit are in clusters at the apices of branches. These clusters

usually consist of a few to many peduncled spikes, racemes or even panicles originating in the axils of the apical bracts which are numerous and in dormant branches which completely cover the apices.

At the beginning of a new season when the trees are starting to sprout, branch apices either produce a new flush of leaves or slowly develop inflorescences (STREY 1973). The leafy branches produced in the previous season are shed before a mature inflorescence is developed. In *C. spicata*, instead of producing an inflorescence, an umbel of fairly slender leafy branches is produced at the apex of a trunk during the first season. Only during the following season are inflorescences formed at the apices of the branches.

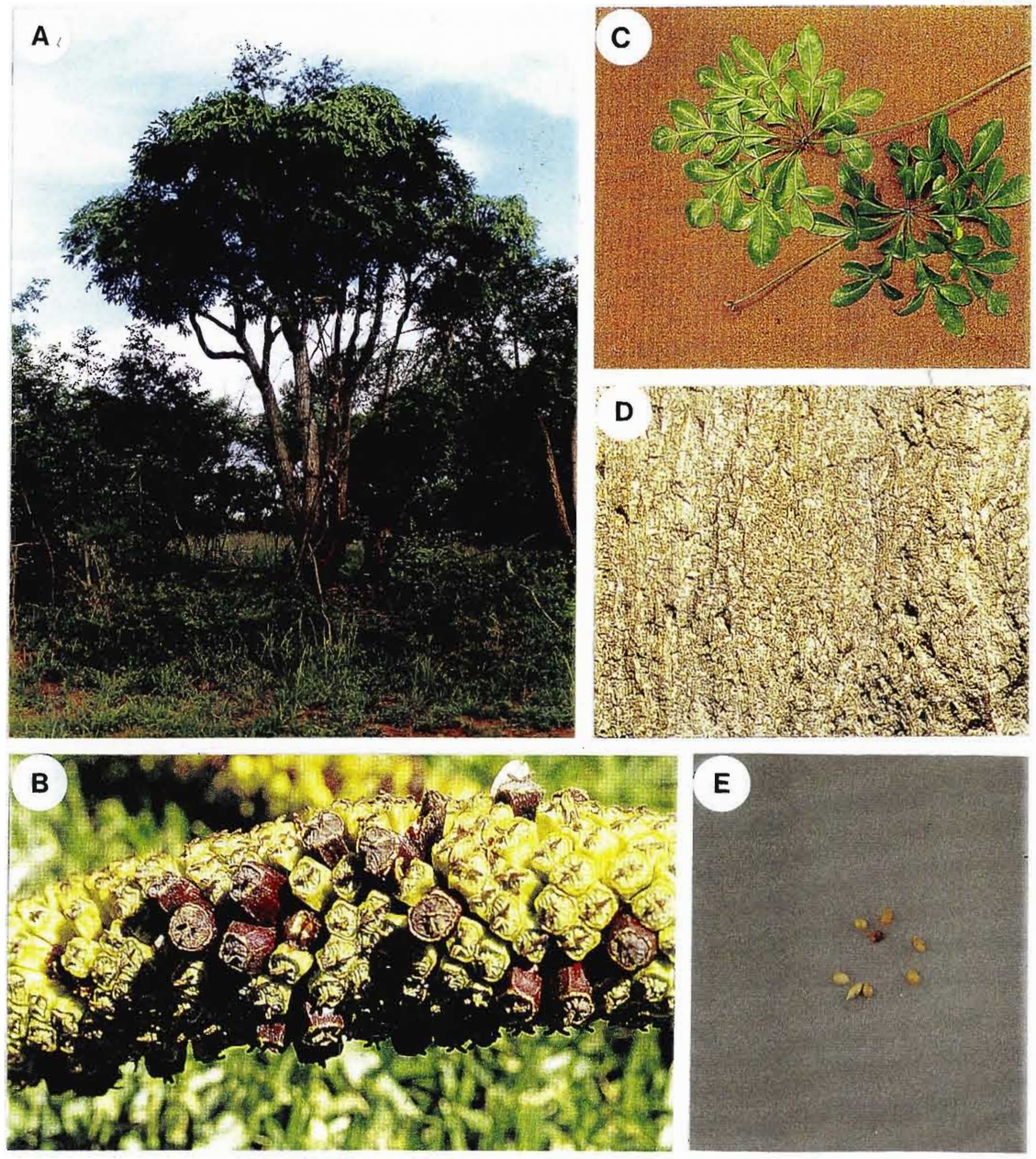


Figure 1. Habit of *C. spicata*: mature tree (A), inflorescence (B), leaves (C) and bark (D). *C. paniculata* seeds (E)

1.2 A historical background on tissue culture methods

One of many fundamental problems in the universe is the question of the origin of form and function which man has made many efforts to understand. SCHWANN (1839) suggested a theory of totipotency which stated that if cells of an organism are alike, within the genetic pattern and totipotent, then the difference in their behaviour is caused by their differential responses to the external environment and the influence of the cells surrounding them. If their behaviour is induced internally, it means that some of its totipotency was lost at some point in the sequence of its temporal association with other cells (WHITE 1943). The concept of totipotency led to the requirement of techniques to reduce an organism to its constituent cells and to study these cells as elementary "organisms".

It was only at the beginning of the 20th century that the great Austrian botanist, HABERLANDT (1902), made a statement that paved the way for the study of single cells. To put it in his exact words, " To my knowledge, no systematically organised attempts to culture isolated vegetative cells from higher plants in simple nutrient solutions have been made. Yet, the results of such culture experiments should give some interesting insight to the properties and potentialities, which the cell as an elementary organism possesses. Moreover, it would provide information about the inter-relationships and complimentary influences to which cells within a multicellular whole organism are exposed".

HABERLANDT was the first to culture isolated fully differentiated cells as early as 1898. The lines cited above are from the English translation of his

classic paper presented in 1902. In this paper he described the results of these classic pioneering experiments (BOJWANI and RAZDAN 1983). The discipline that HABERLANDT outlined is presently known as "tissue culture". In his work, he was confronted with three problems.

Firstly, he perceived the concept of growth hormones, which he called "growth enzymes". He felt that these are released from one type of cell and stimulate growth and development in other cells. The only problem with postulate is that there was no natural nutrient that could be extracted from a plant and used for the successful cultivation of its cells and for subsequent analysis. Secondly, plant cells were covered with a rigid cell wall, which upon removal produced a naked protoplast that is subject to shock and thus prevented cells from adhering to substratum. Thirdly, growth in the plant cell was restricted to certain regions, leaving other parts inert, as far as capacity for continued cell manipulation is concerned. This in turn posed a problem of choosing materials to work on for the researcher (WHITE 1943). Therefore, the choice of materials mainly depended on the plant part that is chosen for manipulation. These plant parts included leaves, stems, roots (usually bulbs), shoot meristems and axillary buds.

HABERLANDT foresaw the possibility of cultivating isolated cells. Until recently, only small pieces of tissue could be grown in culture. Further progress was made by MUIR (1953) when he transferred callus tissues of *Tagetes erecta* and *Nicotiana tabacum* to liquid medium. By agitating the cultures on a shaker, it was possible to break callus tissue into small cell aggregates. Various aspects such as condition of plant material, source of

explant, decontamination method and culture conditions need a great deal of attention before a researcher can conduct tissue culture research.

1.2.1 Tissue culture systems for *in vitro* propagation

In tissue culture systems where the objective is vegetative propagation, investigators generally categorise the developmental sequence of events into three or four stages. Stage I occurs following the transfer of an explant onto a culture medium. Enlargement of the explant and/or callus proliferation occur during this stage. Stage II is characterised by a rapid growth increase of organs and/or the induction of adventitious organs or embryos that occurs sometime following subculture onto medium with or without altered growth factor concentration ratios. Stage III pertains to rooting or hardening to relate some tolerance to moisture stress or confirming a degree of resistance to certain pathogens. But most of all it involves the conversion of the plant from a heterotrophic stage to the autotrophic stage. Stage III development may therefore require subculture onto new medium. In addition to these stages, stage 0, for stock plant growth under controlled conditions was later included to ensure healthier and more uniform explants. In summary, the stages today recognised are:

Stage 0, the preparation of stock plants under hygienic conditions (DEBERGH and MAENE 1981);
Stage I, establishment of the aseptic culture;
Stage II, multiplication of propagula; and
Stage IIIa, preparation for re-establishment in soil (MURASHIGE 1974a, b, 1976, 1978).

Motivation for the development of stage 0 was due to the inability to find plants that yielded non-contaminated explants after the usual decontamination procedure for tissue culture. It is important to use healthy stock plants as starting material. Stock plants in this stage are grown under controlled conditions prior to *in vitro* culture to ensure healthier explants and a uniform response to the first reaction. The type of watering regime also has an influence on the quality of stock plants. Plants have to be given water directly to the pot or by capillary action. It is apparent therefore that the impact of stage 0 is not limited to the sanitary situation of the explants in Stage I, but it also influences the rate of survival (DEBERGH and MAENE 1981).

In micropropagation, there are important points to be considered before conducting experiments. These include the condition and source of explants, methods of decontamination, and the medium that is going to be used for propagation. The success or failure of micropropagation of tree species often depends upon the condition of the plant material at the time of collection (BONGA and VON ADERKAS 1992). This is particularly true when explants are obtained from trees grown in the field. The physiological conditions of tissues vary with seasons, position within the tree, and climatic factors. Each of these conditions can affect the manner in which tissue responds in culture.

In addition, natural environmental factors influence the level of microbial contamination of the tissues (BONGA and VON ADERKAS 1992). Because of the unpredictable effects of the outdoor environment on the physiological condition of plants, many researchers prefer to use material

grown in the greenhouse (DIRR and HEUSER 1987). In the greenhouse, plant vigour can be maintained by using optimal light, temperature and fertilization schedules (BONGA and VON ADERKAS 1992). Because of this, explants of greenhouse-grown plants are often less contaminated than those from the field.

Decontamination of juvenile material is generally not difficult. However, if older material is used, contamination of explants is sometimes a serious problem, unless the tree produces juvenile sprouts (BONGA and VON ADERKAS 1992). In field grown trees insects deposit spores. Spraying the material with insecticides and fungicides can therefore reduce the contamination. A fungicide that is frequently used is Benlate, the concentrations of which can be varied according to the type of the plant material that will be used for tissue culture.

This decontamination step is preceded by dipping the plant material in alcohol because microorganisms, which can be trapped in leaf axils or at the base of hairs, may never come in contact with the sterilant because of air bubbles entrapped in these positions. Another surface sterilant, mercuric chloride ($HgCl_2$) is also used to decontaminate actively growing shoots of several hardwood species. The disadvantages are that this chemical poses health risks and disposal problems. Mercuric chloride may also kill the plant material if treated for long periods; hence it is not used frequently. Hydrogen peroxide has been used successfully to decontaminate seed coats. Inside the seed coats are the embryos that are not affected by the decontamination process. Advantages of hydrogen peroxide are that it does not leave any toxic residues and sometimes

stimulates seed germination as it partly scarifies the seed covering(s) (BONGA and VON ADERKAS 1992). Apart from using these sterilants, often the only way to obtain contamination-free cultures is by culturing apical shoot meristems (BONGA and VON ADERKAS 1992). Table 1.1 summarizes surface decontaminants used in tissue culture.

After explants have been subjected to decontamination procedures, they are manipulated under sterile conditions and transferred to sterile culturing vessels containing nutrient media for growth and development.

Table 1.1 *Surface decontaminants used to treat explants prior to culture*

Decontamination agent	Concentration	Treatment time (min)
Antibiotics	4-50 mg l^{-1}	30-60
Bromine water	1-2%	2-10
Calcium hypochlorite	9-10%	5-30
Ethanol/*Iso*propanol	70%	Quick dip
Hydrogen peroxide	10-2%	5-15
Mercuric chloride	0.1-1%	2-10
Sodium hypochlorite[1]	10-20%	5-30

[1] Commercial bleach (3.5%)

In plant cell, tissue and organ culture, nutrient media are an important factor (BONGA and VON ADERKAS 1992). Media design is particularly

difficult because of interactions of various chemicals with the tissues. Furthermore, environmental factors such as light intensity and quality, photoperiod, temperature, and pH and whether the medium is solid or liquid influence the nutrient and tissue interaction. Most media have been developed slowly by continual trial and error until optimum media are obtained.

However, there are species which grow well on a wide range of different media; thus, there are no precise optimal media for these species (BONGA and VON ADERKAS 1992). GEORGE, PUTTOCK and GEORGE (1987) (in Table 2, Vol. 1) have divided the components of plant tissue culture media into four major categories: macroelements, microelements, vitamins and amino acids/amines. There are some classical, widely used media, such as WHITE (1943), MURASHIGE and SKOOG (1962), GAMBORG, MILLER and OJIMA (1968) B5, LITVAY, JOHNSON, VERMA, EINSPAHR and WEYRAUCH (1981), and LLOYD and McCOWN (1980) Woody Plant Medium. Many of the other media used are minor or major modifications of these.

Table 1.2 *The constituents of a modified MURASHIGE and SKOOG (1962) medium*

Stock Number		Salts	Mass /1litre(g)	Vol. of Stock (ml) in Final vol. of 1 L
MACRO-NUTRIENTS	1	NH_4NO_3	1650	10
	2	KNO_3	1900	20
	3	$CaCl_2.2H_2O$	440	10
	4	$MgSO_4.7H_2O$	370	10
	5	NaFeEDTA	37.3	10
	6	KH_2PO_4	170	10
MICRO-NUTRIENTS	7	H_3BO_3 $MnSO_4.7H_2O$ $ZnSO_4.7H_2O$	6.2 22.3 0.84	10
	8	$Na_2MoO_4.2H_2O$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$	0.25 0.025 0.025	10
VITAMINS	9	Glycine Pyridoxin HCL Thiamin HCL Niacin	2.0 0.05 0.025 0.05	10
SUPPLEMENTS		Sucrose Myo-inositol	- -	30.0 g l^{-1} 0.10

Culture media used must be modified in such a way that they can promote shoot or root initiation, depending on the type of culture that is being made. The most popular medium used is MURASHIGE and SKOOG (1962) basal medium (Table 2.2). It can be used at full strength or can be diluted depending on the optimal nutrient requirements of a certain species.

Culture media can either be used in liquid form or can be solidified. To solidify a medium, a gelling agent is added. Gelling agents are usually polymers that set on cooling after autoclaving. Agar is the most widely used substance and is extracted from seaweeds. Concentrations of 0.8-1% are normally used. It is necessary to use purified forms as agar varies from batch to batch. Agarose can also be used but it is expensive. Although polyacrylamide gels can also act as solidifying agents, they are highly toxic to plant cell cultures therefore they are not used. Gellan gums e.g. gelrite (0.2%) are becoming increasingly used despite being more expensive per unit of weight than agar. Whatever gelling agent is chosen, it may have effects on the rates of diffusion of molecules through medium to the explant (CURREL, JAMES, LEACH, PATMORE, VAN DAM-MIERAS, DE JEU and DE VRIES 1993). Agar has an effect on the water potential of the medium, and the concentration chosen affects the development of stomata and occurrence of hyperhydricity.

For large-scale operations, cell suspension cultures are preferred over cultures grown on solid nutrient media. Liquid cultures generally grow faster, require less handling, and are easier to automate. All of the mentioned media can be used alone or can be supplemented with plant growth regulators that will stimulate growth of tissues *in vitro*. Growth

regulator concentrations in the culture medium are critical to the control of growth and morphogenesis (LEOPOLD 1987).

Five principal classes of plant growth regulators have been recognised namely: auxins, gibberellins, cytokinins, abscisic acid and ethylene. By relevance, only auxins, cytokinins and gibberellins will be discussed in detail since they were used in this project. Of these five classes, auxin and cytokinins play a major role in the vegetative propagation of plants *in vitro*. Failure to respond to growth regulators is often a problem when explants from mature trees are cultured. The problem decreases when explants from juvenile material are used. To control growth and differentiation, growth hormones are added to the nutrient medium or are supplied as a short pulse before culture. Once absorbed, the hormones interact with growth regulators synthesized by the cells, and a growth response will follow.

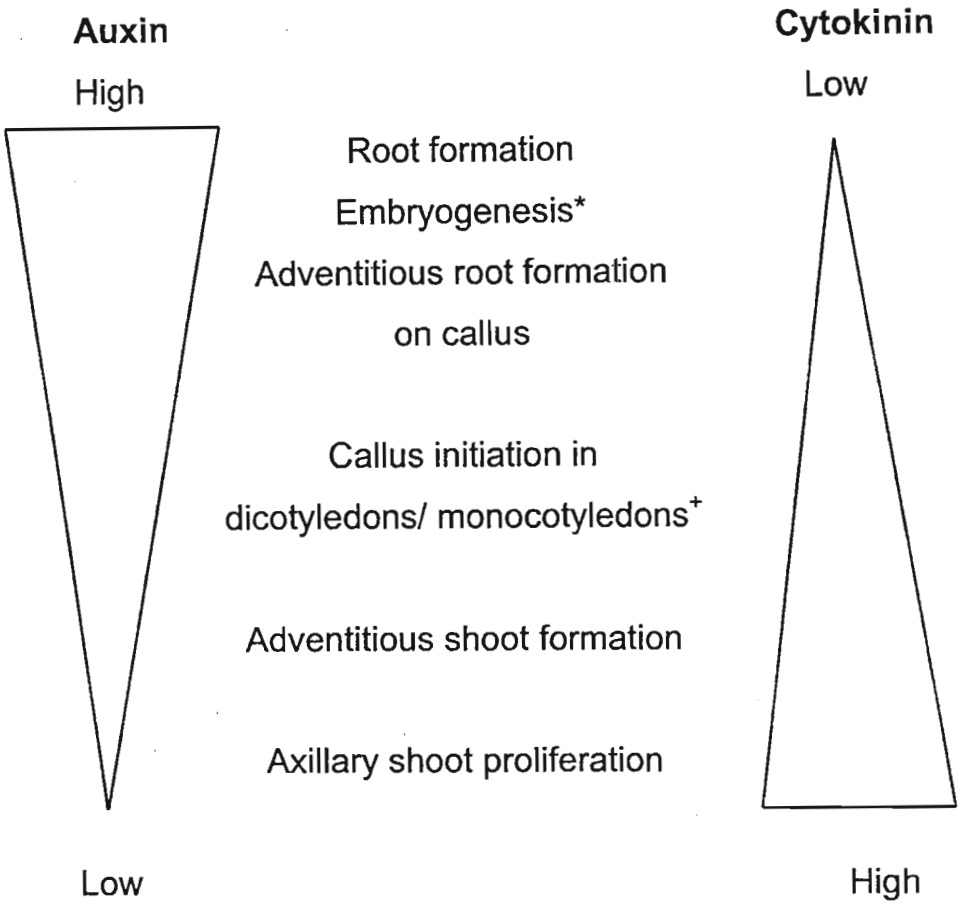
Auxins are involved in cell division, elongation and cell wall synthesis (LEOPOLD 1987). The principal natural auxin is indole-3-acetic acid (IAA) but because of its instability, it is not frequently used in tree tissue culture. During media preparation approximately 40% of IAA in MS medium is destroyed by 20 min autoclaving (NISSEN and SUTTER 1988). Due to this problem with IAA, other auxins such as indole-3-butyric acid (IBA) and ∝-naphthaleneacetic acid (NAA) are generally preferred for use in tissue culture. Auxins generally stimulate adventitious root formation. When used in correct combination with cytokinins, they can stimulate shoot or callus formation.

One of the main functions of cytokinins in tissue culture is induction of adventitious shoots. They are also used to release axillary buds from suppression by dominance, thus initiating shoot proliferation. Cytokinins are sometimes required for the induction of somatic embryogenesis. MATSUTA and HIRIBAYASHI (1989) could only induce embryogenesis when 2,4-dichlorophenoxyacetic acid (2,4-D) was combined with benzyladenine (BA) in cultures of *Vitis vinifera*. Commonly used cytokinins are isopentenyladenine (iP), zeatin, BA, and kinetin. Of these, iP and zeatin are naturally occurring. The cheapest and the most active cytokinin is BA (BONGA and VON ADERKAS 1992). Benzyladenine did induce adventitious shoots 10-20 times more efficiently than with iP in embryonic explants of *Pinus strobus* (FLINN, WEBB and GEORGIS 1986).

When cytokinins are combined with auxins in different ratios, the effect on the growth of an explant may be pronounced. Figure 2 is a summary of the promotory effects of cytokinins and auxins on *in vitro* grown explants. SKOOG and MILLER (1957) put forward the concept of hormonal control of organ formation. They showed that the differentiation of roots and shoots in tobacco pith tissue cultures is a function of the auxin: cytokinin ratio. They further showed that changing the relative concentrations of the two substances in the medium could regulate organ differentiation.

High concentrations of auxin promote rooting, whereas high concentrations of cytokinins enhance shoot production. At equal concentrations of auxin and cytokinin, the tobacco pith tissue tended to grow in an unorganised fashion. This concept of hormonal regulation of organogenesis is now applicable to most plant species. However, the exogenous application of

growth hormones for a particular type of morphognesis varies, depending on the endogenous levels of these substances in the tissue in question.



* Organogenesis in monocotyledons is often promoted in a medium without auxin or by reducing the auxin concentration.

+ In monocotyledons, callus induction may require high levels of auxin, cytokinins may be unnecessary or unimportant.

Figure 2. A diagrammatic presentation of the effects of cytokinins and auxins on *in vitro* grown explants (CURREL, JAMES, LEACH, PATMORE, VAN DAM-MIERAS, DE JEU and DE VRIES 1993).

Gibberellins generally control cell elongation, bud breaking and seed germination, and have been associated with flowering. A few reports were made on GA_3 having beneficial effects in cultures of tree species. In low concentrations it caused inhibition of bud development of *Auraucaria* explants (BURROWS, DOLEY, HAINES and NICKLES 1988), but had no effect on cotyledon cultures of *Pinus radiata* (BIONDI and THORPE 1982) and meristem cultures of *Sequoiadendron gianteum* (MONTEUUIS 1987).

Having established an aseptic culture, it has to be maintained in a controlled environment for growth and development. Temperature, light and gaseous exchange are the most important factors in the physical environment for cultures. Based on whether the original material is tropical or temperate, environmental conditions have to be determined. Cultures of some tropical tree species are very temperature sensitive. For example, in a study on teak culture, 28°C was used as the incubation temperature (HARRY and THORPE 1994). Embryogenic cultures of *Elaesis guineeusis* sustain chilling injury when the temperature decreases below approximately 20°C (CORBINEAU, ENGELMANN and CÔME 1990). For temperate poplar species, a range of 22 to 26°C was appropriate, depending on the explants being cultured. Tissues of some tree species grow faster *in vitro* if the night temperature is kept 5 to10°C below the day temperature (BONGA and VON ADERKAS 1992).

Light is involved in a variety of functions in plant development. It serves as the energy source in photosynthesis. Light also affect cell differentiation and development. Fluorescent (Gro-Lux, or similar type) tubes are the most commonly used source of light. These fluorescent tubes have some

red in their spectrum, which is useful in photosynthesis. They also produce less heat, making them the preferred light source in confined spaces such as growth chambers.

Photoperiod is also important because some species require specific light and dark periods. For example, shoots of *Pseudotsuga menziesii* grew when exposed to 16 or 24 h of light per day, with 16 h exposure giving a better growth rate (BONGA and VON ADERKAS 1992). *Rhododendron* shoot cultures elongated more and produced more shoots in second subculture, when placed for 16 h per day under cool, white fluorescent light than when maintained under continuous light. Periods of darkness sometimes influence *in vitro* growth. Morphogenesis in embryo or cotyledon cultures of some conifers is stimulated by several weeks' exposure to darkness at the start of culture (WEBB and STREET 1977).

1.2.2 A brief outline of the principal methods of micropropagation

Plants can be micropropagated via direct or indirect methods. Figure 3 represents the principal methods of micropropagation. In direct propagation, explants are derived from the mother plant, and then placed onto a growth medium which will promote growth. Shoot apex or meristem cultures are normally used as a means of direct propagation. The first successful shoot apex culture was performed by LOO (1945) using *Asparagus*. Shoot apex culture is often confused with apical meristem culture. True meristem culture is important in the development of pathogen free plants (MURASHIGE 1974a, QUAK 1977). In apical meristem culture, the dome of the apical bud which is about 0.1 to 0.3 mm long is used as an

explant. For shoot apex culture, the shoot tip that is used is about 3 mm in length and includes two to three leaf primordia. Usually it is difficult to work with the apical meristem because it is very small and very sensitive to sterilants. Apical meristem culture is not suitable for propagation *in vitro* because only one plantlet will arise from a single meristem. It is valuable mainly for academic reasons and rarely for practical needs (MURASHGE 1978). Its excision demands exceptional skill, and survival frequency and plant development of the cultures are considerably poorer than those of larger shoot apices. Moreover, the probability of pathogen elimination is not substantially better than that attainable with the leaf primordium-containing apices (MURASHGE 1978). With shoot apex culture and subsequent subculturing, axillary and multiple axillary shoots can be obtained. This culture is suitable for rapid clonal propagation.

Indirect propagation incorporates the use of various explants from any part of the mother plant that will undergo morphogenesis. Morphogenesis can be either direct or indirect. With indirect morphogenesis, shoot formation or somatic embryos are formed directly on the explant. The shoots formed can be rooted and prepared for planting in soil. Somatic embryos then form somatic seedlings. Indirect morphogenesis involves callus initiation on the explants. The callus can be placed on media which will promote adventitious shoot formation on callus or indirect morphogenesis. Callus can also be placed into suspension cultures and single cells obtained can develop into plantlets.

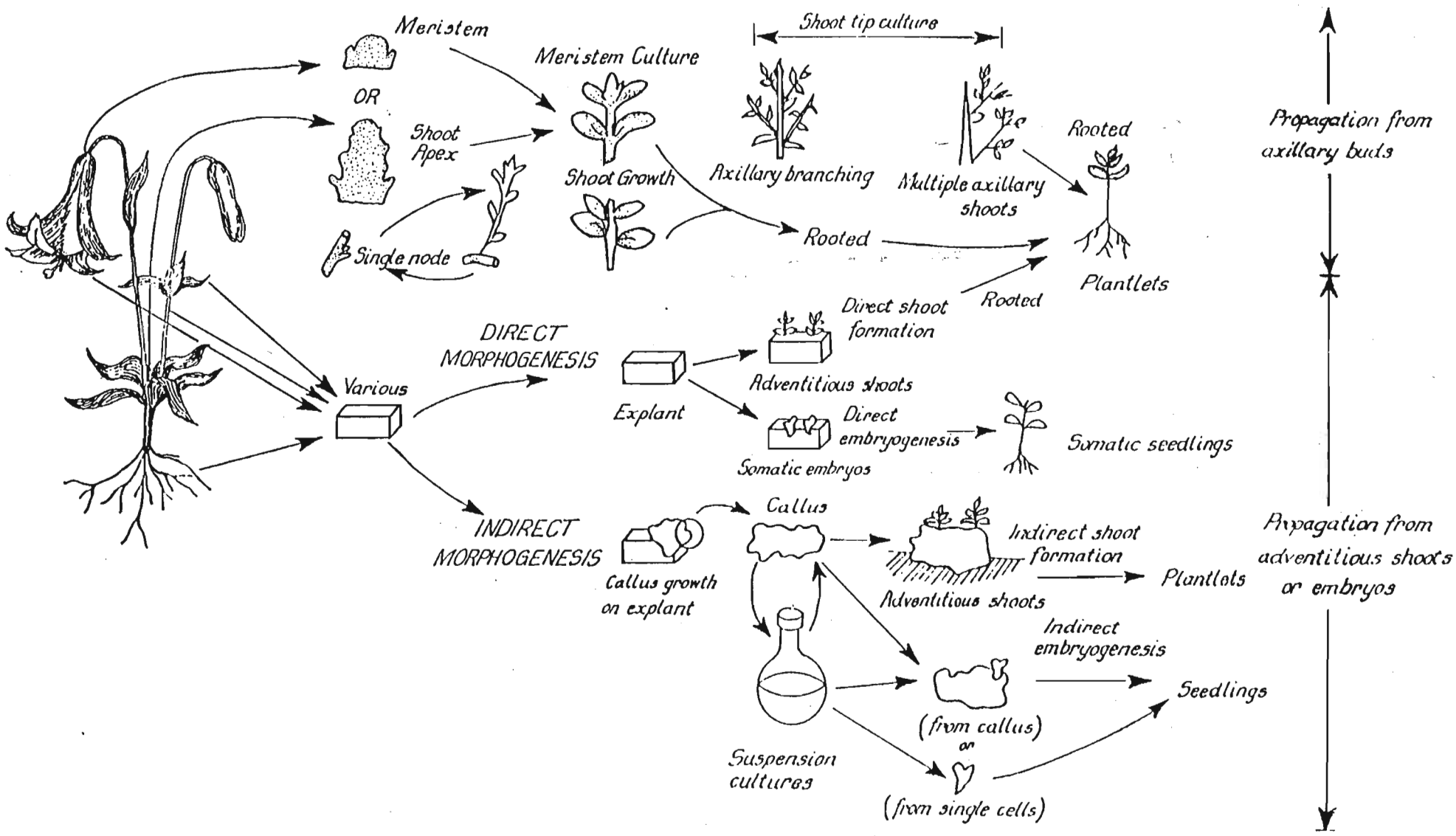


Figure 3. The principal methods of micropropagation (FINNIE 1988).

1.2.3 Tissue culture of the Araliaceae

There is no evidence from a literature search indicating that *Cussonia* has been studied in terms of tissue culture. However, some species belonging to this family, namely *Dysisma pleitha, Aralia cordata, Aralia elata, Acanthopanax sciadophylloides*, and a few species of the *Panax* genus, have been studied in tissue culture. *Panax ginseng*, a medicinal woody plant, has been subjected to a number of tissue culture studies. These include embryonic callus induction, and secondary metabolite production *in vitro*. In *Aralia elata*, studies have been made on callus induction and plant regeneration from leaf explants. Secondary products produced by species of this family that have been widely studied are the saponins (CHOI, PARK and AHU 1990). They were produced by cell suspension cultures.

The effect of nutrients on anthocyanin production in cultured cells of *A. cordata* was determined (SAKAMOTO, SAWAMURA, IIDA, HAJIRO, ASADA, YOSHIKAWA and FURUYA 1993). The medium for callus induction was supplemented with 3% sucrose, 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin.

A three-year juvenile period has hampered conventional breeding programmes for rapid multiplication of *Panax ginseng,* providing a good reason for micropropagation. To achieve this, leaf explants of *Panax ginseng* were used to make rotational suspension cultures in order to determine cell types produced in culture. The cells were fixed in preparation for scanning electron microscopy and amongst them were parenchymatous cells, embryonic and embryogenic cells (DING, CHEN,

XIANG, HE, YOU, CHEN and DING 1993). Adventitious somatic embryos of *Panax ginseng* were then subjected to rapid multiplication. Zygotic embryos were cultured on MS with 1.0 mg l^{-1} 2,4-D, 0.01 mg l^{-1} kinetin and 30 g l^{-1} sucrose. Embryogenic callus obtained was subcultured onto MS with 1.0 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose at 20 °C with a 16 h photoperiod. Embryonic cells were produced by callus induced from *Pseudomonas quinquefolium* in MS medium supplemented with 1 mg l^{-1} 2,4-D. The genus *Tupidanthus*, which also belongs to the Araliaceae family, has been studied in tissue culture. The studies involved meristem culture for propagation (GEORGE, PUTTOCK and GEORGE 1987).

1.3 Aims and objectives of this study

(i) The aim of this study was to determine the medicinal properties of *Cussonia* species so as to validate their use in traditional medicine. This involved screening of *Cussonia* species for anti-bacterial, anti-inflammatory and anti-malarial activities.

(ii) Considering its medicinal value and wide use, the project was extended to investigate whether *Cussonia* species could be micropropagated by *in vitro* methods as a means of rapidly producing and conserving these plants. This required establishing:

(a) An optimum method for decontamination;

(b) An optimum culture medium for micropropagation; and

(c) Acclimatization of microplants that could then be transferred to the external environment.

CHAPTER 2

SCREENING *CUSSONIA* SPECIES FOR MEDICINAL PROPERTIES

2.1 Introduction

The use of *Cussonia* species in traditional medicine is well known for treatment of ailments caused by infections, inflammation and malaria (Table 2.1). Special preparations of specific plant parts are used for treating particular ailments. However, of interest is the rationale for the use of certain species to treat ailments. Therefore, a need arises to validate whether the preparations of plant parts used to treat various diseases are used because of availability or whether they confer a favourable result as postulated by some traditional healers. Consequently, *Cussonia* species were tested for anti-bacterial, anti-inflammatory and anti-malarial activity, as a means of validating their use. In addition, TLC fingerprinting was performed to establish whether there are differences in the chemical composition of these species.

2.1.1 Anti-bacterial screening

There are a few methods available for anti-bacterial screening of plant extracts. The basic principle of these methods is similar. Bacterial strains are co-cultured with the plant extract. A positive result, the inhibition of bacterial growth, is detected visually or spetrophotometrically.

Table 2.1 *Traditional medicinal uses of* Cussonia *species* (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996; WATT & BREYER-BRANDWIJK 1962)

Species traditionally used	**Plant part used**	**Preparation**	**Ailment Treated**
C. arborea (Hochst ex A. Rich)	Root	Emenagogue	-
C. paniculata (E. & Z.)	Leaf	Decoction (boiled)	Early nervous and mental disease
C. paniculata (E. & Z.)	-	Enema	Colic, dysmenorrhoea
C. spicata (Thunb.)	Bark	Warmed	Relieve cramps, stomach ulcers
C. spicata (Thunb.)	Stem, Root	Decoction	Dysmenorrhoea
C. spicata (Thunb)	Bark, Root	-	Gonorrhea and other venereal diseases
C. spicata (Thunb)	Leaves	-	Indigestion
C. spicata (Thunb)	Root	Powder	Emetic in biliousness
C. spicata (Thunb.)	Root	Infusion	Malaria
C. umbellifera (Sond.)	Bark	-	Malaria
C. umbellifera (Sond.)	Leaves	-	Rheumatism, colic and Insanity

-= Plant part, preparation or ailment not specified in literature

Gram-negative and Gram-positive bacteria are the two types of bacterial strains used in screening procedures. Frequently used strains are the Gram-negative *Escherichia coli*, *Pseudomonas aerugiea*, *Klebsiella pneumonia and* Gram-positive *Micrococcus luteus*, *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus epidermis*, respectively. Although these strains are responsible for common infections, they are safe to work with because they are non-virulent.

In this project, the disc diffusion assay outlined by RASOANAIVO and RATSIMAMANGA-URVERG (1993) was employed. The fundamental principle of the method is that a plant extract of interest or neomycin, which is used as a standard, is applied to filter paper discs and allowed to diffuse into the overlayed medium inoculated with test bacteria. After an overnight incubation, a clear zone of growth inhibition is measured and expressed as the ratio of inhibition relative to the neomycin standard. The size of the zone of inhibited growth is indicative of the degree of anti-bacterial activity of the test extract.

2.1.2 Anti-malarial screening

The *in vitro* anti-malarial test is based on the inhibition of [G-^{3}H]-hypoxanthine uptake by *Plasmodium falciparum* cultured in human blood (RASOANAIVO and RATSIMAMANGA-URVERG 1993). This inhibition occurs in the erythrocytic schizogony, the asexual division of mosquitoes, which occurs in human host red blood cells. Figure 4 represents a simplified cycle of a reproducing mosquito. In the *in vitro* assay, parasites are allowed to reach the mature stage in the presence of a plant extract being tested using fresh human red blood cells. If chloroquine and a given

plant extract are simultaneously combined in a suitable series of concentrations, it is possible to evaluate the influence of the plant extract on the IC_{50} (inhibitory concentration) of the chloroquine against a chloroquine resistant strain of *P. falciparum*.

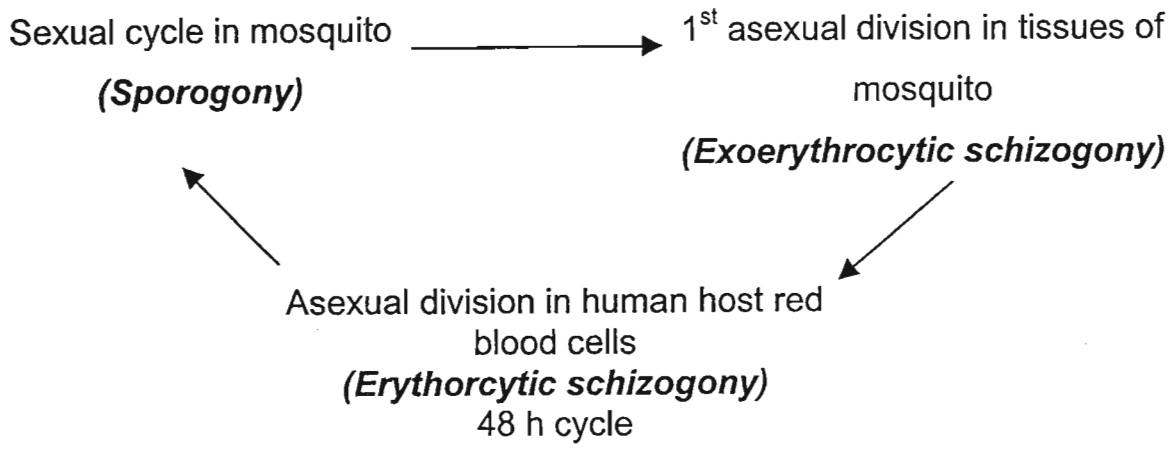


Figure 4. Reproduction cycle of a mosquito infected with *P. falciparum* (RASOANAIVO and RATSIMAMANGA-URVERGA 1993).

2.1.3 Anti-inflammatory screening

Prostaglandins are groups of pharmacologically active lipids, widely distributed in mammalian tissues and body fluids (WEEKS 1973). These chemical compounds are involved in the complex processes of inflammation. Prostaglandins are known to be responsible for the sensation of pain. The cyclooxygenase enzyme also catalyzes the inflammation process. Therefore, during anti-inflammatory screening, plant extracts are screened for cyclooxygenase inhibitors.

2.1.3.1 Chemical nature of prostaglandins

Primary prostaglandins are unsaturated hydroxy-acids with a 5-membered ring in a 20-carbon skeleton (WEEKS 1973). Trivial names are by letter and number. There are over a dozen prostaglandin variations in double bonds and hydroxyls.

2.1.3.2 Possible physiological and pathological roles of prostaglandins.

The local formation of prostaglandins and their rapid inactivation suggests a physiological role not as a classical systemic hormone but instead a local role, within or near the tissue in which they are formed (WEEKS 1973). It is suggested that since their release is associated with some other stimulus, they function as modulators rather than mediators. PGAs may be an exception, since they are relatively resistant to inactivation by the lungs.

The most widely studied prostaglandins are PGE_1, PGE_2, $PGF_{2\alpha 1}$ and PGA_1. The primary E- and F_α- prostaglandins are powerful smooth muscle stimulants, while the other prostaglandins are relatively inactive in this respect. Primary PGEs and PGAs have a similar depressor action in all species studied. However, in cats and rabbits $PGF_{2\alpha}$ has a depressor action while it has an antagonistic effect in dogs, rats, monkeys, and humans (WEEKS 1973). Furthermore, prostaglandins are implicated in some pathological conditions such as male sterility and spontaneous abortion. Low levels of seminal prostaglandins have been correlated with some cases of male sterility. Spontaneous abortion has also been associated with a premature elevation of prostaglandins in the amniotic fluid. Prostaglandins formed in burned skin may contribute to vascular

reactions to burns. WEEKS (1973) also suggests that prostaglandins are amongst the mediators formed in the inflammatory reaction, and a pathological role is supported by the non-steriodal anti-inflammatory agents also being powerful inhibitors of prostaglandin synthetase.

2.2 Materials and Methods

2.2.1 Collection and preparation of plant material

The leaves, bark, stem and roots of *Cussonia spicata* and *Cussonia paniculata* were collected from the University of Natal Botanic garden, Pietermaritzburg. Leaves and bark of *Cussonia umbellifera* (=*Schefflera umbellifera*) were collected from KwaZulu-Natal Nature Conservation Services, Pietermaritzburg. The plant material was collected and stored in paper bags and then placed in a 50°C oven to dry. The dry material was ground into fine powders and stored in airtight plastic honey jar containers at room temperature. The experiments in this section were limited by the amount of plant material available.

2.2.2 Preparation of plant extracts

For screening purposes, each extract was prepared by extracting 2 g powdered plant material with 20 ml of water, ethanol or ethyl acetate for 30 min in an ultrasound bath. The extraction mixtures were centrifuged or filtered, the supernatants decanted and then air-dried. The residues were resuspended in water, ethanol and ethyl acetate respectively to an assay-specific concentration. Dry extract residues were otherwise stored at room temperature until required. Plant extracts were screened using assays outlined below.

2.2.2.1 Disc diffusion assay

Leaf, bark, stem and root extracts of *C. spicata* and *C. paniculata* were tested for anti-bacterial activity using the disc diffusion assay (RASOANAIVO and RATSIMAMANGA-URVERG 1993). Only leaf and bark extracts of *C. umbellifera* were screened for anti-bacterial activity as stem and roots were not available.

Small broth cultures of the test bacteria were prepared the night before, to have saturated cultures for inoculation the following morning. Using the overnight cultures to inoculate from, broth cultures (3 ml) of the test bacteria to inoculate the top layer agar (Mueller-Hinton (MH) agar - 5 ml per plate) were prepared. Base plates were prepared by pouring MH agar into sterile Petri dishes (9 cm) on the laminar flow bench. Agar was allowed to gel (10 ml agar per plate). On the day of the assay, the top agar was autoclaved separately in Schott bottles for each bacteria and then kept at 48 °C, to maintain its molten state. Working on the laminar flow bench, 10 µl of plant extract at a concentration of 50 mg ml^{-1} was pipetted onto pre-autoclaved filter paper discs and allowed to air dry.

At the same time, discs with neomycin diluted to 200 µg ml^{-1} (10 µl per disc) were prepared in the same way and were used as control discs. After 5-6 h when broth cultures of the test bacteria have become turbid, they were added to the top layer agar in the Schott bottles, which had been held at 48 °C. This dilution gave about 10^5 cells per ml media (the OD_{600} of ~ 1 corresponds to 10^8 cells per ml in a saturated culture). Immediately after inoculating the agar with the bacteria, it was poured over the base plates to form a homogenous top layer. Dried filter paper discs were now placed on

top of the inoculated top layer agar, with the antibiotic control disc in the center and four plant extract discs equidistant around the centre one. The plates were incubated at 37°C overnight.

2.2.2.2 Anti-malarial screening

Bark extracts of *C. spicata*, *C. umbellifera* and *C. paniculata* were tested for anti-malarial activity. Only the bark extracts were tested because preparations of these are used in traditional medicine. The extracts were tested at 50, 100 and 200 µg ml^{-1}. The following anti-malarial assay was performed by Dr. E. A. Prozesky at the University of Pretoria, South Africa: The South African isolate (PfUP1) of the malaria parasite *Plasmodium falciparum* was used in the bioassays. For continuous *in vitro* culturing, a slightly modified version of the Trager and Jensen method was employed (TRAGER and JENSEN 1976, HOPPE 1993).

The wash medium consisted of 10.4 g RPMI 1640 L-glutamine, 5.94 g HEPES buffer, 4.0 g D-glucose, 44 mg hypoxanthine, 5% sodium hydrogen carbonate and 4 mg of gentamycin dissolved in 900 ml deionised sterile water. For use as culture medium, this wash medium was supplemented with 10% human serum of a positive blood group after heat inactivation at 56°C for 20 min. The parasite culture was then suspended in 10 ml of this culture medium, in a 75 ml culture flask (Sterilin). The culture, consisting of parasites and culture medium was then further supplemented with fresh, uninfected human erythrocytes with an O+ blood group.

To determine the activity of extracts against *P. falciparum* in an accurate *in vitro* assay, a slightly modified version of the parasite lactate

dehydrogenase assay was used (MAKLER, RIES, WILLIAMS, BANCROFT, PIPER, GIBBINS and HINRICHS 1993). The experiment was done in triplicate with a 1% parasitemia and a 5% hematocrit and incubated at 37°C for 48 h in 96-well, flat bottomed microtiter plates in 5% O_2, 5% CO_2, 90% N_2. After 48 h, a duplicate plate was prepared with 100 µl Malstat™ reagent [133 ml Triton X-100, 1.33 g lactate, 0.44 g TRIS buffer and 44 mg 3-acetylpyrimidine adenine dinucleotide (APAD)]. This solution was made up to 200 ml in distilled water and added to each plate together with 25 µl developing dye solution [160 mg Nitroblue tetrazolium (NBT) and 8 mg Phenazine ethosulphate (PES) to 100 ml Millipore water] and 10 ml from the incubated plate. The duplicate plate was incubated for 20 min in the dark and read with an ELISA plate reader at 620 nm.

2.2.2.3 Cyclooxygenase (COX-1) assay

A slightly modified procedure of the WHITE and GLASSMAN (1974) method was used to screen for prostaglandin-synthesis inhibitory activity (JÄGER, HUTCHINGS and VAN STADEN 1996). The co-factor solution (3 mg adrenaline and 3 mg reduced glutathionine, dissolved in 10 ml 0.1 M Tris buffer, pH 8.2) was incubated on ice for 15 min. Co-factor solution was combined with the cyclooxygenase enzyme solution. Sixty microlitres (60 µl) of this mixture was added to 20 µl of the samples or standard solution being tested (20 µl aqueous samples and 17.5 µl H_2O + 2.5 µl ethanolic solution). The concentration of the plant extract samples was diluted to a concentration of 8 µg ml^{-1}. The standard solution in this case was 2.5 µl of a 8 X 10^{-4} M ethanolic indomethacin solution +17.5 µl H_2O. Twenty microlitres of [^{14}C]-arachidonic acid (16 Ci/mole, 30 mM) were added and the assay mixture was incubated at 37°C for 10 min. Adding 10 µl of 2 N

HCl terminated the reaction. A blank was kept in the ice bath and after the incubation, 5 µl of a 0.2 mg ml^{-1} carrier solution of unlabelled prostaglandins (PGE_2: PGF2 1:1) was added.

The prostaglandins were separated from the unmetabolized arachidonic acid by column chromatography. Silica suspended in eluent 1 (hexane-dioxane-acetic acid 350:1 50:1) was packed into Pasteur pipettes up to a 3 cm mark. After adding 1 ml of eluent 1 to all the samples, they were applied to the columns and 4 ml of eluent 1 was added to each column to elute arachidonic acid. Prostaglandins were collected by means of applying 3 ml of eluent 2 (ethyl acetate-methanol 85:15) to each column. After mixing with scintillation solution, 30 min was allowed for chemoluminescence to disappear and then radioactivity was counted. All samples were in duplicate. The percentage inhibition was calculated using the following equation:

$$1 - \left(\frac{DPM_{sample} - DPM_{background}}{DPM_{blank} - DPM_{background}} \right) \times 100$$

2.2.3 Thin Layer Chromatography

The concentration of the plant extracts was adjusted to 25 mg ml^{-1}. Twenty microliters of each extract were applied using a Pasteur pipette with a tapering end as a thin band (1 cm wide) along the origin of a silica based thin layer plate. The TLC plates were then placed in a small chromatography tank containing a solvent mixture (10 ml final vol) suitable for separating a particular extract. The chromatogram was left to run until the solvent front was ±1 cm from the top of the plate. The lid of the tank was smeared with Vaseline® to ensure an airtight compartment.

Plates were subsequently dried in a 110°C oven for 3-5 min and viewed under UV-light at 254 nm and 366 nm. To develop the invisible compounds, TLC plates were sprayed using a vacuum spraying apparatus with an anisaldehyde stain (465 ml ethanol; 5 ml glacial acetic acid; 13 ml concentrated sulphuric acid; 13 ml para-anisaldehyde). The plate was then heated in a 110°C oven for 5-10 min until it turned pink. Solvent systems were chosen by virtue of their ability to separate as many compounds as possible. After a number of trial runs, the solvent systems finally used for separation were:

Leaf extracts of *C. paniculata*, *C. spicata,* and *C. umbellifera.*

Toluene: Ethyl Acetate 8:2

Bark, Stem and Root extracts of *C. paniculata*, *C. spicata,* and *C. umbellifera.*

Hexane: Ethyl Acetate 4:1

The R_f values were then determined by measuring from one centre of the spot to the origin. The R_f value is defined as the ratio of the distance travelled by the compound to the distance travelled by the solvent. The latter is measured from the origin to the solvent front.

2.3 Results and Discussion

2.3.1 Anti-bacterial activity

After an overnight incubation at 37°C, a clear inhibition zone was visible

around the neomycin paper discs and some of the plant extract discs. Table 2.2 shows the ratio of the inhibition zone of the *C. spicata* extracts (50 mg ml^{-1}) to the inhibition zone of the reference, neomycin (200 µg ml^{-1}) against the test bacteria. Generally, water extracts did not show anti-bacterial activity, especially bark extracts.

Table 2.2 *Ratios of inhibition zone of* C. spicata *bark and root extracts to the inhibition zone of neomycin*

Bacterial strain	**Gram status (- or +)**	**Plant part analyzed**	**Extracts**		
			Water	**EtOH***	**EtAc***
E. coli	-	Bark	0.00	0.15	0.20
		Root	0.06	0.00	0.00
P. aerugiea	-	Bark	0.00	0.09	0.13
		Root	0.00	0.10	0.15
K. pneumonia	-	Bark	0.00	0.20	0.10
		Root	0.19	0.14	0.19
M. luteus	+	Bark	0.00	0.27	0.09
		Root	0.25	0.10	0.14
B. subtilis	+	Bark	0.20	0.09	0.09
		Root	0.18	0.05	0.00
S. aureus	+	Bark	0.00	0.10	0.22
		Root	0.09	**0.50**	**0.50**
S. epidermis	+	Bark	0.00	0.00	0.02
		Root	0.12	0.24	0.44

EtOH* = Ethanol, EtAc* = Ethyl Acetate

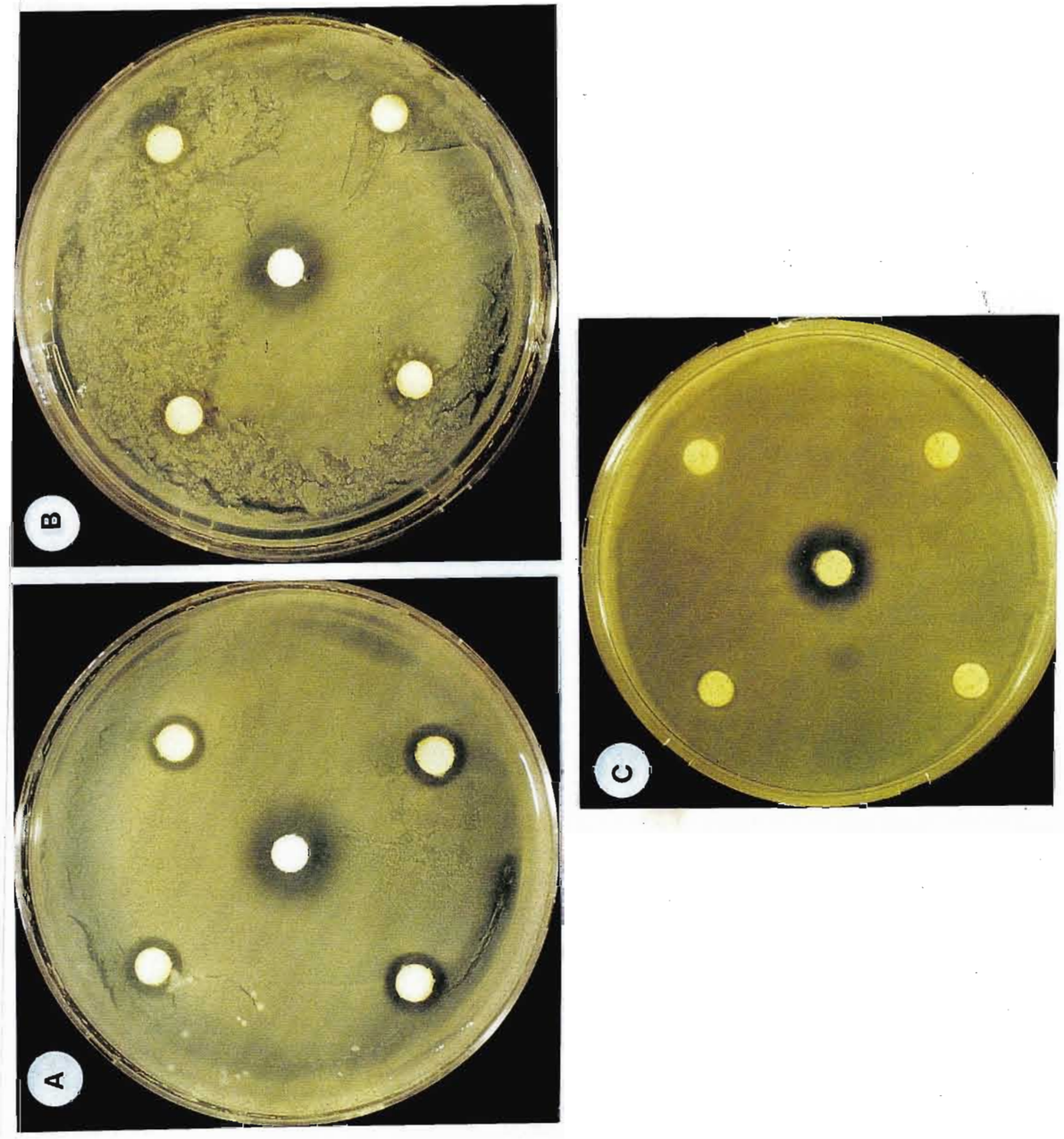


Figure 5. Anti-bacterial activity of *C. spicata* root (A) and bark (B) extracts against *Staphylococcus aureus*. *C. paniculata* bark extract showed no anti-bacterial activity against *S. aureus* (C).

Ethanol and ethyl acetate root extracts showed the highest inhibition ratio (0.50) against *Staphylococcus aureus*. Figures 5A and 5B represents anti-bacterial activity of *C. spicata* root and bark extracts against *S. aureus* after an overnight incubation at 37°C in the disc diffusion assay, respectively. *C. paniculata* extracts did not show any anti-bacterial activity (Figure 5C).

All three species were screened for anti-bacterial activity but only *C. spicata* showed significant activity. This result validates the use of *C. spicata* in traditional medicine. The root and bark decoction of this species is used in southern Africa as a treatment for gonorrhea and other venereal diseases. *C. spicata* is easily accessible to traditional people because it is the most abundant species of the genus in southern Africa. There is limited information available about the dosage of medicine given to sick patients by traditional healers. However, the amount of activity displayed by bark and root extracts does not suggest great amounts of active compounds that could possibly be isolated. Because of low activity, there is probably less chance of toxicity. Furthermore, the amount of activity of these extracts could provide some indication regarding the intensity of the healing ability of the extracts. Possibly, the patients treated with the root and bark preparations usually have minor infections. Although it is not referred to in the literature, this species could be used in conjunction with other medicine to give patients the desired result. This is usually the case in many other traditional, as well as western medicines.

2.3.2 Anti-malarial activity

Table 2.3 presents the percentage inhibition against a Chloroquine

Resistant Plasmodium strain of *Plasmodium falciparum.* Not much activity was found with *C. spicata* and *C. paniculata* bark extracts. However, *C. umbellifera* showed a high inhibitory activity (100 %) with the ethanol and ethyl acetate bark extracts at a concentration of 200 µg ml^{-1}. Even at lower concentrations of 50 µg ml^{-1}, ethanol and ethyl acetate bark extracts of *C. umbellifera* showed an activity of 45 and 55%, respectively. Therefore, the results suggest that *C. umbellifera* has a higher concentration of active compounds than *C. spicata* and *C. paniculata.* Literature shows that both *C. spicata* and *C. umbellifera* are used traditionally to treat malaria (Table 2.1). Traditional people used roots of *C. spicata* and the bark of *C. umbellifera.* Therefore, the screening validates the use of these two species in traditional medicine. The limitation in the number of extracts to be screened did not allow screening of other plant parts. In the future, other parts of the plant could be screened to investigate whether they show anti-malarial activity. The activity of the extract depends on the concentration of the active compound(s). Those that showed high activity could be used to isolate the active compounds.

Table 2.3 *Percentage inhibition of* Plasmodium falciparum *by bark extracts of* C. spicata, C. umbelliffera *and* C. paniculata

Plant species	Extract	Concentration (µg ml^{-1})		
		50	100	200
C. paniculata	water	-	-	10
	EtOH	-	-	30
	EtAc	-	-	20
C. spicata	water	-	-	20
	EtOH	-	-	35
	EtAc	-	-	-
C. umbellifera	water	-	-	-
	EtOH	45	80	100
	EtAc	55	90	100

Chloroquine IC_{50} = 150 ng ml^{-1}, - = No activity

2.3.3 Anti-inflammatory activity

C. spicata showed less anti-inflammatory activity than *C. paniculata* and *C. umbellifera* in the COX-1 assay (Table 2.4). Generally, ethanol and ethyl acetate extracts were more active than water extracts. In the COX-1 assay indomethacin (5 µM in final assay vol), the standard, inhibited cyclooxygenase to a level of 78%. Extracts with a minimum activity of 50% and 70% for water and ethanolic/ethyl acetate extracts respectively, were considered as active.

Although *C. paniculata* showed inhibition against cyclooxygenase, the highest activity was displayed by *C. umbellifera* leaf and bark extracts

(Table 2.4). Both *C. paniculata* and *C. umbellifera* are used for treatment of diseases associated with inflammation such as rheumatism, dysmenorrhoea, colic, as well as diseases associated with the nervous system (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996; WATT & BREYER-BRANDWIJK 1962). Customarily, a decoction of the leaves is a widely used preparation to treat these diseases (WATT & BREYER-BRANDWIJK 1962). However, the screening of other plant parts showed that the bark and stem extracts of *C. paniculata* were also active. The stem and roots of *C. umbellifera* were not available for testing.

C. spicata is also used for treating diseases associated with inflammation such as dysmenorrhoea, stomach cramps and stomach ulcers (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). Some *C. paniculata* and *C. spicata* bark extracts gave a negative result in the COX-1 assay. JÄGER, HUTCHINGS, and VAN STADEN (1996) suggested that a negative result does not fundamentally mean that the plant is without anti-inflammatory activity. The active compound(s) could work at other sites in the complex process of inflammation.

Referring to recorded literature (Table 2.1), all three species are used by traditional healers to cure diseases that are caused by the complex process of inflammation. However, the species mostly used is not specified. Therefore, it can be speculated that two factors, readily availability and intensity of the healing ability, mainly affect the choice of the species. Availability becomes a problem when traditional healers have harvested and depleted what was readily available to them. Alternatively, they are bound to travel long distances to obtain plant material they need. Additionally, patients could also influence traditional healers by demanding

medicines known best to work for healing a particular illness.

Further work that could possibly emerge from this is isolation of active compounds, especially from *C. paniculata* and *C. umbellifera*. Attention could be given to those plant parts, which showed high activity of more than 70% in the bioassay.

Table 2.4 *Percentage cyclooxygenase inhibition of extracts from* Cussonia *species*

Species	**Plant part**	**% inhibition**		
		Water	**Ethanol**	**Ethyl Acetate**
C. paniculata	Leaves	29	80	58
	Bark	-	42	78
	Stem	27	85	80
	Roots	32	15	38
C. spicata	Leaves	15	56	13
	Bark	35	-	-
	Stem	51	19	35
	Roots	35	27	38
C. umbellifera	Leaves	39	92	80
	Bark	19	93	92

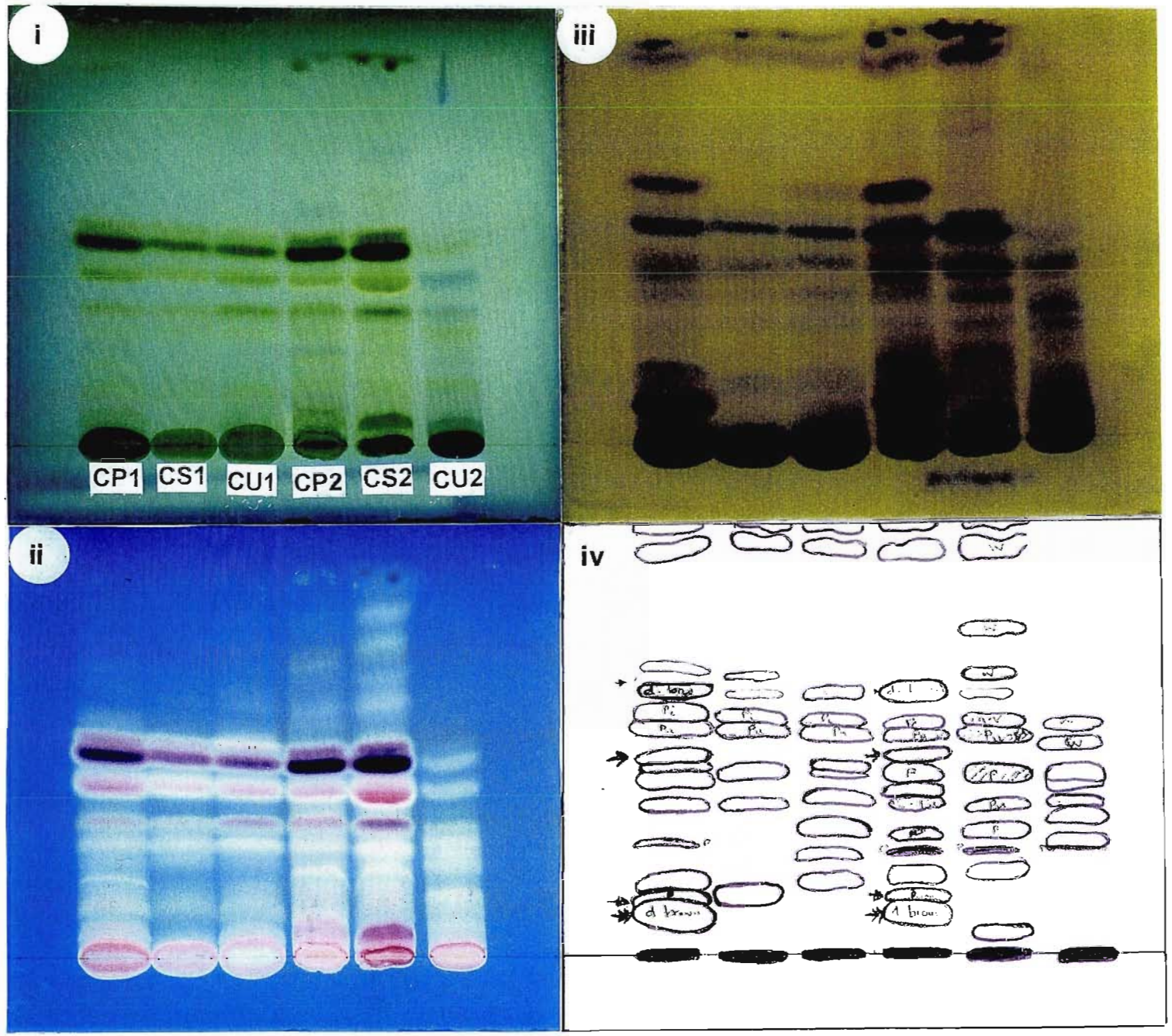
Indomethacin (0.5 µg) 78%

- = Negative percentage inhibition

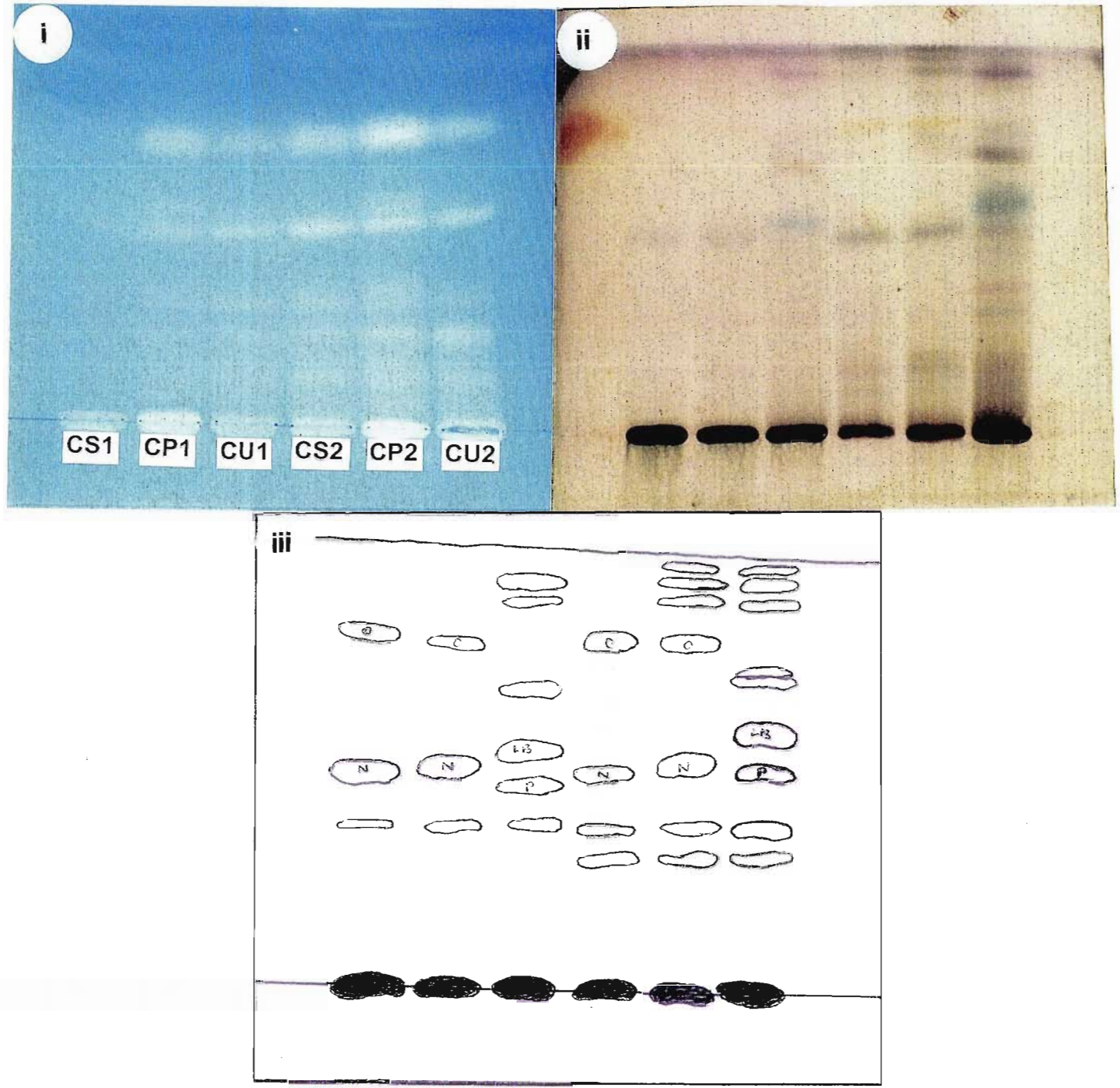
Figure 6. Thin layer chromatograms of ethanol and ethyl acetate extracts of *C. paniculata*, *C. spicata* and *C. umbellifera*. (A) Leaf at (i) UV 254 nm; (ii) UV 336 nm; (iii) anisaldehyde stain; and (iv) diagrammatic presentation of the TLC. (B) Bark at (i) UV 336 nm; (ii) anisaldehyde stain; and (iii) diagrammatic presentation of the TLC. (C) Stem at (i) UV 336 nm; (ii) anisaldehyde stain; and (iii) diagrammatic presentation of the TLC. (D) Root at (i) UV 254 nm; (ii) UV 336 nm; (iii) anisaldehyde stain; and (iv) diagrammatic presentation of the TLC. Lane labels represent the following:

CP = *C. paniculata,* CS = *C. spicata,* CU = *C. umbellifera*
1 = ethanol extracts, 2 = ethyl acetate extracts

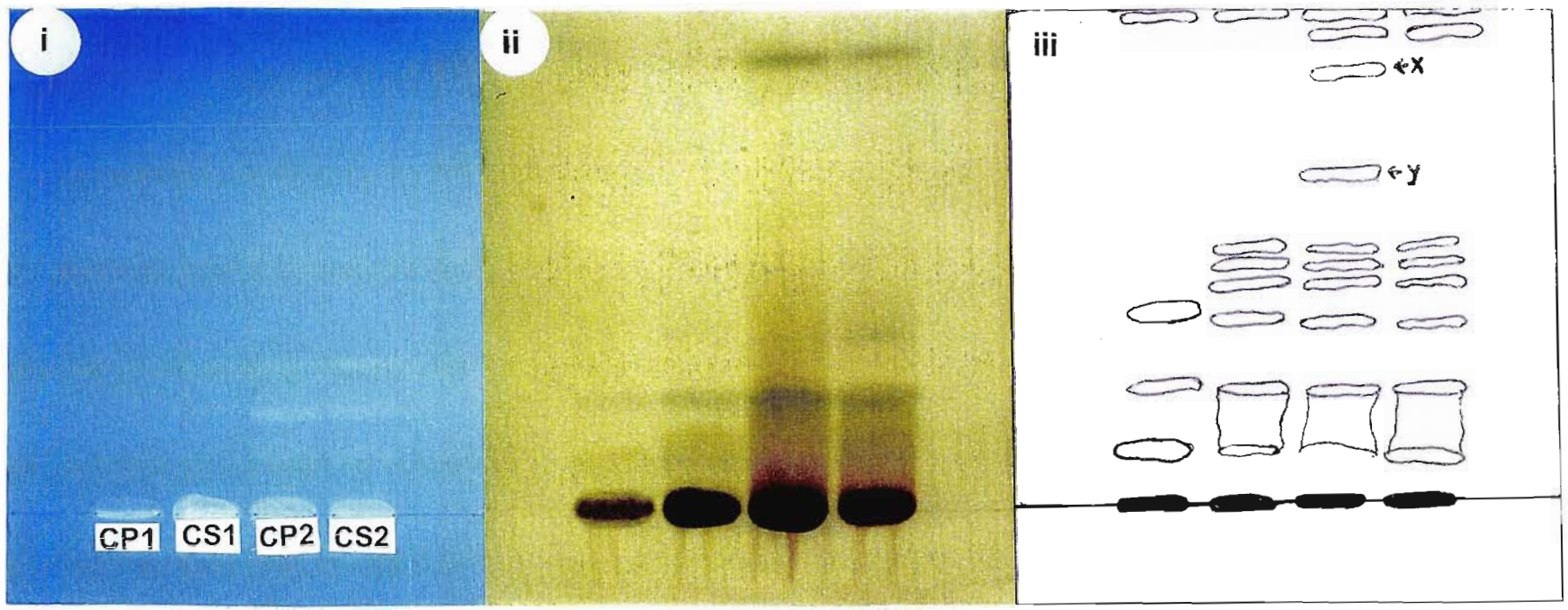
A



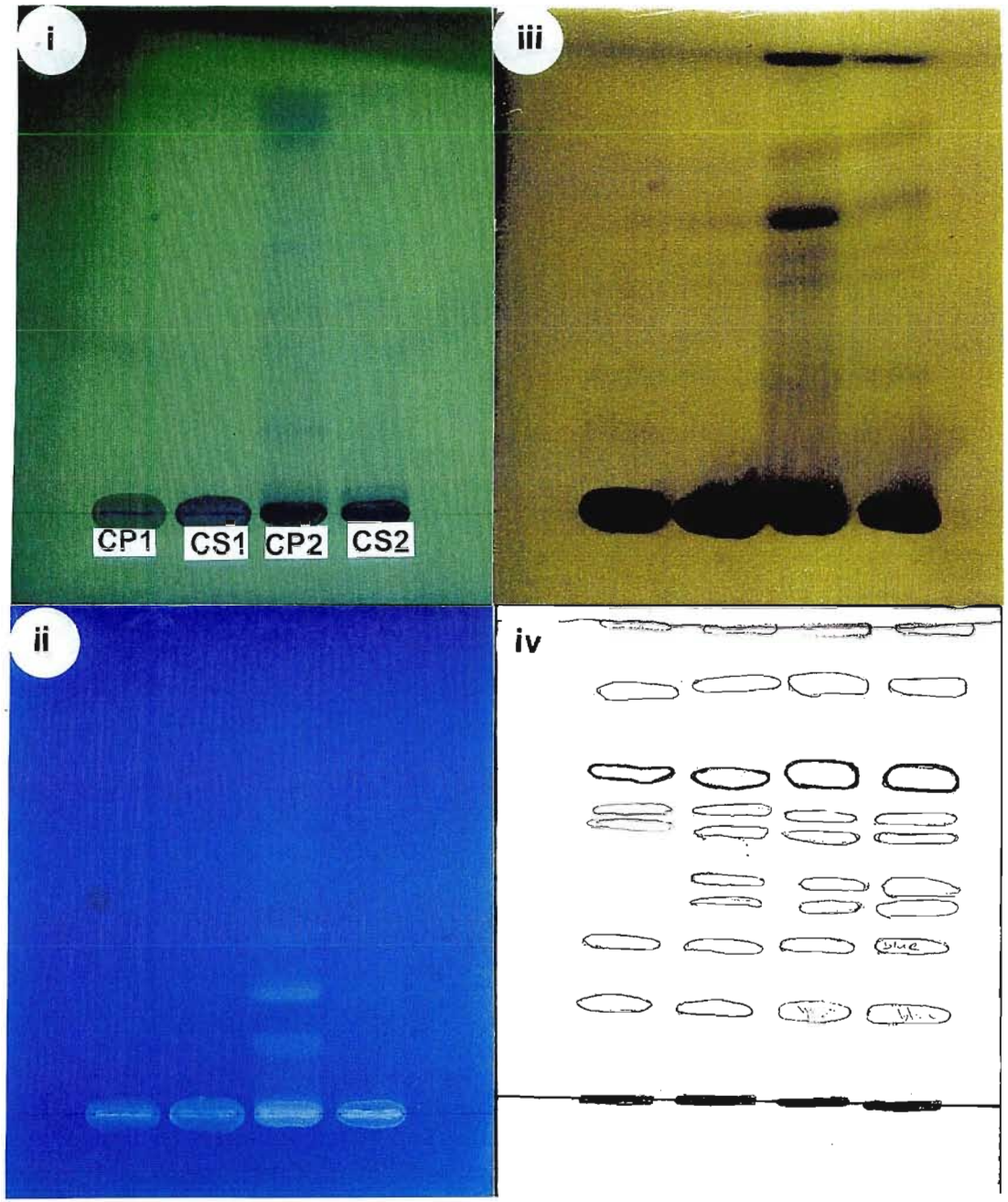
B



C



D



2.3.4 Thin Layer Chromatography

There is an apparent difference in the chemical composition of the leaf extracts of the three species (Figure 6A). *Cussonia paniculata* (lane CP1 and CP2) has three compounds that *C. spicata* and *C. umbellifera* do not have. These compounds are situated at R_f 0.09 (dark brown), 0.13 (fluorescent pink) and 0.47 (pink/orange), respectively. *Cussonia spicata* (lane CS1 and CS2) and *C. paniculata* have a fluorescent green compound at R_f 0.4 that is not present in *C. umbellifera*. Apart from these differences, all lanes have bands with similar R_f values and colours in the chromatograph.

All leaf extracts of the three species were subjected to screening for biological activity. Positive results were obtained with the COX-1 assay, where *C. umbellifera* was more active that the other species. Consequently, *C. umbellifera* is expected to be different somewhat in terms of chemical constituents from the other species. However, the chromatogram shows a similarity in compounds for all species except the above mentioned differences. The *C. paniculata* and *C. spicata* compounds mentioned above could therefore be some other compounds which are not necessarily involved with anti-inflammatory activity. Anti-inflammatory activity could be a result of different compounds or a combination of compounds rather than a single one.

Bark extracts of the three species were separated and Figure 6B represent the TLC plate. *C. umbellifera* (CU1 and CU2) had three compounds that the other species did not have. These compounds are situated at R_f 0.47 (purple), 0.60 (light blue), and 0.69 (purple).

Bark extracts were tested for their medicinal properties and they showed positive results in the anti-bacterial and anti-inflammatory assays. *C. umbellifera* ethanol extracts exhibited the highest percentage inhibition (100%) against *P. falciparum* at 200 µg ml^{-1} while *C. spicata* and *C. paniculata* showed inhibition of <50%. *C. umbellifera* ethanol extracts had the highest inhibition against prostaglandins (93%). The lack of, or less activity in *C. spicata* and *C. paniculata* may be due to the absence of the compounds that *C. umbellifera* has.

Looking at the separation of stem extracts of *C. spicata* and *C. paniculata* (Figure 6C), CS2 lacks two bands, x (R_f 0.58) and y (R_f 0.84), that CP2 had. *C. paniculata* ethyl acetate stem extract exhibited 80% inhibition against prostaglandins while *C. spicata* extract gave 35% inhibition. The absence of these compounds could mean that the stem extract of *C. spicata* does not have compounds necessary for anti-inflammatory activity. Apart from this difference, the stem extracts of *C. paniculata* and *C. spicata* seem to have similar compounds. For ethanol extracts, it is difficult to conclude whether CP1 had the highest activity because both CS1 and CP1 lacked bands x and y.

Root extracts (Figure 6D) showed bands of similar R_f values and colours for both species. The similarity in the number of bands and the way they separate suggests that these two species have similar compounds. In the disc diffusion assay, *C. spicata* root extracts showed anti-bacterial activity while root extracts of *C. paniculata* did not show activity. There is no difference in the chemical composition visible in the TLC plate between these two species. Therefore, the bands already on the TLC plate could represent the compounds responsible for anti-bacterial activity in *C.*

spicata. A combination of these bands could possibly bring about anti-bacterial activity. Alternatively, it could mean that the compounds did not develop during staining, therefore were invisible.

Although the TLC gives some indication of the difference in the chemical constituents of these species, it is not satisfactory to conclude that the different compounds are the ones responsible for different biological activities. Therefore, it is not adequate to deduce that TLC fingerprinting validates the use of these species in traditional medicine but can be securely used to distinguish between the species of the same genus, especially if plant parts have to be identified.

Future prospects would therefore require a further analysis of the plant extracts, which showed activity. The compounds need to be isolated, identified and re-tested for activity, a task beyond the scope of this study.

CHAPTER 3

MICROPROPAGATION OF *CUSSONIA* SPECIES

3.1 Introduction

In vitro propagation has many advantages over conventional propagation methods such as rooting of cuttings and grafting procedures (HARRY and THORPE 1994). Although these traditional methods are effective, they have some limitations. The material for cuttings has to be juvenile. According to HARRY and THORPE (1994) the following are some of the advantages of propagating *in vitro*: (1) Like *Cussonia*, some trees do not produce enough vegetative shoots for rooting of cuttings or for grafting. The few vegetative shoots produced by these trees are enough for *in vitro* vegetative propagation because small explants are required for culture. (2) Pathogens can often be removed from specimens or clones and the procedures in tissue culture are kept under aseptic conditions. (3) Environmental conditions of plant growth can be optimised. (4) There is a great possibility of micropropagating for commercial purposes. (5) *In vitro* methods also ensure rapid propagation on a large scale.

In the previous Chapter, the importance of *Cussonia* species in traditional medicine and their horticultural potential was outlined. This part of the project to determine whether the species of this genus can be propagated *in vitro* was motivated by the success of screening tests in validating their use in traditional medicine.

There are certain problems that researchers experience when optimising regeneration of plants by *in vitro* means. One problem that is usually observed to some extent is hyperhydricity (vitrification) of the shoots. Hyperhydricity of the shoots was observed in this study. It affected rooting and acclimatization of the plantlets.

3.1.1 Symptomatology of hyperhydric plants

The term hyperhydricity has been adopted to describe the occurrence and nature of the glassy shoot syndrome (DEBERGH, AITKEN-CHRISTIE, COHEN, GROUT, VON ARNOLD, ZIMMERMAN and ZIV 1992). An abnormal morphological appearance and physiological function of organs and tissues in tissue culture, as well as the transition from liquid to solid state can be described as hyperhydricity. Symptoms characterizing hyperhydricity are not identical in all plants (DEBERGH, AITKEN-CHRISTIE, COHEN, GROUT, VON ARNOLD, ZIMMERMAN and ZIV 1992). However, one can assume that changes in anatomy, morphology, and physiology begin as soon as an explant is placed in culture. Visual symptoms occur after a certain period under certain culture and explant conditions. These symptoms can be avoided by controlling the composition of the culture medium, the environmental conditions in which the culture containers are maintained and the quality of the cultured explants.

3.1.1.1 Morphological and anatomical characteristics of hyperhydric shoots

The morphological and anatomical characteristics described by DEBERGH, HARBAOUI and LEMEUR (1981) include an increase or decrease in stem diameter. Leaves are usually thick, elongated,

wrinkled, and/or curled, brittle, translucent, with a reduced or hypertrophied surface. The leaf colour can be abnormal. Initiation of roots on such shoots is usually difficult. Additionally, leaves have a reduced number of palisade layers (BRAINERD, FUCHIGAMI, KWIATSKOWKI and CLARK 1981), large intercellular spaces in the mesophyll cell layer (PAQUES and BOXUS 1987), and defective epidermal tissue. EARLE and LANGHANS (1975) observed that the leaves could have less surface wax or wax of a different crystalline structure. The vascular connection between roots and stems could also be disturbed (GROUT and ASTON 1977).

DEBERGH, AITKEN-CHRISTIE, COHEN, GROUT, VON ARNOLD, ZIMMERMAN and ZIV (1992) reported that external factors evoke hyperhydricity when other conditions in the culture system are not optimized. These external factors include the explant, medium, container, and incubation environment. For example, benzyladenine will induce hyperhydricity when cultures are also stressed by other factors, such as a high water retention capacity in the headspace of the container.

3.1.1.2 Treatment and remedies to control hyperhydricity

Solidified media with a higher concentration of a gelling agent or a gelling agent with higher gel strength have been used successfully in the past to avoid this disorder (DEBERGH, HARBAOUI and LEMEUR 1981; ZIV, MEIR and HALEVY 1983). In addition, lowering the cytokinin concentration or substituting one for another (usually replacing benzyladenine with a different cytokinin) has also been useful (PAQUES and BOXUS 1987). The type of container used must permit gas exchange and prevent the build-up of gases such as ethylene in the headspace of the container.

3.2 Materials and Methods

3.2.1 Preparation of an aseptic working environment

The laminar flow bench was generously sprayed with 70% alcohol and wiped down with cotton wool before each and every use. Autoclaved instruments (scalpels, tweezers etc.) were kept in a glass bottle with absolute alcohol and they were flamed before and after each excision to remove the alcohol. The flame was enough to remove any trace contaminants on the instruments. Hands and arms of the worker were sprayed with 70% alcohol before working on the bench and periodically while working. The mouths of culturing tubes and bottles were flamed before and after each transfer so as to eliminate contaminants.

3.2.2 Micropropagation of *Cussonia paniculata*

3.2.2.1 Plant material

Seeds of *Cussonia paniculata* (Eckl. & Zeyh.) were purchased from Kirstenbosch Botanical gardens, Cape Town in January 1999. The seeds were stored in an airtight jar at 4°C.

3.2.2.2 Seed decontamination

Seeds of *C. paniculata* were treated with 70% alcohol for 1 min. Under sterile conditions, intact seeds were treated with 3.5% NaOCl (full strength household bleach) for 15 min. The seeds were rinsed in three changes of sterile distilled water with a 5 min soak in the last change. Working with care to avoid injuring the embryos, the seed coats were removed from some of the seeds. The excised embryos were rinsed again in

sterile distilled water before being placed on the medium.

3.2.2.3 Media and supplements

Both excised embryos and intact seeds were germinated on hormone-free MS medium supplemented with 3% (w/v) sucrose. The pH was adjusted to 5.8 with 1N HCl and 0.75 M NaOH before solidifying with 0.3% gelrite. Nodal explants derived from seedlings were placed on full strength MS medium supplemented with 3% (w/v) sucrose, pH 5.8, 0.3% gelrite and various concentrations of BA ranging from 0 to 3 mg l^{-1}. Multiple shoots were excised and individually transferred to a root-inducing medium. This medium was full strength MS medium supplemented with various concentrations of IBA and NAA ranging from 0 to 1.75 mg l^{-1}. There were 15 replicates for each concentration for both cytokinin and auxin supplemented media. Zygotic embryos were also excised and cultured on MS supplemented with a combination of 2,4-D (1 - 3.5 mg l^{-1}) and BA (0.5 – 2 mg l^{-1}). Plantlets from this medium were transferred to MS medium without hormones for further development. Shoots without roots were subcultured on to 0.75 mg l^{-1} IBA for root production.

3.2.2.3.1 Controlling hyperhydricity

The bases of shoots were cut once through with a sharp sterile scalpel. Shoots were initially placed on MS medium supplemented with 1 mg l^{-1} NAA. Before roots were visible, shoots were transferred to MS without growth hormones. The gelling agent was changed from 3 g l^{-1} gelrite to 8 g l^{-1} agar.

3.2.2.4 Culture conditions

All cultures were incubated in a growth room at 25±2°C. The source of continuous light was provided by cool white fluorescent tubes (23 μmol m^{-2} s^{-1}), with a photoperiod of 16 h light: 8 h dark.

3.2.2.5 Acclimatization

Gelrite or agar (in case of plantlets transferred to this medium) was rinsed off the rooted *in vitro* grown plantlets. The plantlets were planted in culture bottles with vermiculite saturated with half strength Hoagland's nutrient solution. The lid of the jars was slightly open to facilitate gaseous exchange. These jars were returned to the above-mentioned culture environment. After two weeks, the plantlets were planted in individual pots in a mixture of potting soil and vermiculite (1:1)(v/v) and transferred to the mist house.

The transplanted plantlets were immediately irrigated with half-strength Hoagland's nutrient solution. These plants were maintained under mist house conditions for 30 days and were thereafter transferred to a green house. After this transfer, the plants were no longer irrigated with the nutrient solution. Once a day on a three-day rotation they were irrigated with water applied straight to the pot.

each decontamination period, there were 20 repetitions. After the treatment, explants were washed three times with sterile distilled water to rinse off the decontamination agent. In this second trial of decontamination, the treatment with Benlate was prolonged to 15 min. A last step was also added to this procedure. The explants were soaked in a 50 mg l^{-1} ascorbic acid solution for 15 min. After decontaminating the plant material, excision took place under sterile conditions.

3.2.3.3 Explant source and size

Shoot-tips of *C. spicata* were excised from mother plants by cutting off approximately 1 cm of the shoot-tip. The meristematic shoot apex (0.4 cm) was carefully isolated. These shoot apices were removed using a sharp sterile scalpel under a dissecting microscope. Shoot-tips were then placed on a contamination-free, wet Whatman® filter paper in an autoclaved glass petri dish while other explants were being prepared. Alternatively they were inoculated to the culture medium immediately after excision.

3.2.3.4 Media and supplements

Decontaminated shoot-tips were placed onto Murashige and Skoog (1962) (MS) medium supplemented with 3% sucrose and various concentrations of auxins and cytokinin. The pH was adjusted to 5.8 using 1 N HCl and 0.75 M NaOH prior to solidifying with 8 g l^{-1} agar. The culture tubes containing the medium were autoclaved at 121°C for 20 min. Media were allowed to cool to room temperature before transferring the explants. The hormone combinations used were as follows: BA (0 -2 mg l^{-1}) and NAA, IBA or IAA (0 -1 mg l^{-1}) with presence of 1 mg l^{-1} GA_3 in all the hormone

combinations.

3.2.3.5 Culture conditions

After inoculation on to the medium, the cultures were incubated in a growth room at 25±2°C. The source of continuous light was provided by cool fluorescent tubes (23 μmol m^{-2} s^{-1}) with a photoperiod of 16h light: 8h darkness.

3.3 Results and Discussion

3.3.1 Micropropagation of *C. paniculata*

3.3.1.1 Seed decontamination and germination

After a period of 30 days, a survival rate of 70% was recorded for seeds of *C. paniculata* with excised embryos (Figure 7). Intact seeds failed to germinate under the same culture conditions. The failure to germinate may be a result of seed dormancy, which is possibly caused by embryo coverings. Embryo coverings may restrict gaseous exchange, water uptake, and mechanical growth of the embryo, possess water-soluble inhibitors in the embryo coverings and promote dormancy as a result of a failure to mobilize embryonic food reserves (BRADBEER 1988). Underdevelopment and undifferentiated embryos, a block of nucleic acid and protein synthesis, deficiency of plant growth substances, and the presence of inhibitors may also cause seed dormancy (BRADBEER 1988). In subsequent experiments seed coats were removed prior to culture. *In vitro* germinated seedlings provided aseptic nodal explants for initiation of multiple shoots (Figure 8A). For many woody angiosperm species,

adventitious or axillary shoots are formed from seedling explants, including nodal stem segments. Response of the explants vary from species to species, time of isolation and position on the seedling (BONGA and VON ADERKAS 1992). Customarily, regeneration from mature *ex vitro* material is favoured rather than starting with seeds because it provides clones of the parent plant. The seed contains an embryo, which during the fertilisation process may have undergone genetic changes. Thus, regenerated plants from seedling explants (organogenesis) are unlikely to be true clones of the mother plant.

Zygotic embryos produced callus on MS medium supplemented with BA and 2,4-D (Table 3.1). The callus was globular in appearance, which later became greenish in colour (Figure 9A). After 25 days, the callus was subcultured to MS without plant growth regulators. In this medium roots were produced. Later on (after 10 days) shoots were observed (Figure 9B). The mass production of plantlets from the callus suggests indirect morphogenesis. Further growth and development of plantlets occurred in the medium without plant growth regulators. Some shoots did not develop roots in the medium supplemented with a combination of 2,4-D and BA. These shoots were subcultured on to MS medium supplemented with 0.75 mg l^{-1} IBA for root initiation (Figure 9C). No signs of abnormalities were observed on these shoots. Several species have been propagated from subcultured callus (BONGA and VON ADERKAS 1992). However, this can lead to genetic instability. Some auxins, in particular 2,4-D and the ones related to it are strong promotors of callus formation. It is suggested that these should, therefore be avoided or be used only at low concentrations for shoot induction. The plantlets obtained were prepared for acclimatization.

3.3.1.2 Shoot initiation from nodal explants

Nodal explants from *in vitro* germinated seedlings were subcultured onto MS supplemented with various concentrations of BA (Figure 10). Most angiosperm tree species are successfully regenerated on MS or its modifications with various combinations of cytokinins, mostly BA. *C. paniculata* was also successfully regenerated on this medium. All the explants produced shoots on medium containing 2 or 2.5 mg l^{-1} BA (100 %) after a 30-day incubation period. Multiple shoots were also produced with 2, 2.5 and 3 mg l^{-1} BA (Figure 8B from left to right). The highest number (3.5) of shoots per nodal explant was recorded with 2.5 mg l^{-1} BA (Figure 10). Shoots were also produced in MS medium without growth hormones.

The shoots were produced as a cluster of shoots per nodal explant (Figure 8B far right). Each of these shoots had several nodes, which were individually separated and placed on MS medium lacking plant growth hormones. The separated shoots in turn produced another flush of shoots. *C. paniculata* showed rapid adventitious shoot proliferation within a short space of time. From a single nodal explant there is a potential of regenerating a minimum of 20 plantlets. With subsequent cutting of the axillary nodes and subculturing, this number may increase. This regeneration is dependent on the hormone level in the medium. The results show that most of the regeneration occurred on MS medium supplemented with 2 or 2.5 mg l^{-1} BA rather than when no hormones were present. After 35 days, the shoots were ready for transfer to root inducing medium.

3.3.1.3 Root initiation

The shoots produced were excised and transferred individually to a root-inducing medium. For this experiment, various concentrations of IBA and NAA (Figure 11) were used. After a period of 30 days roots were observed. A concentration of 0.75 mg l^{-1} IBA yielded the highest number of shoots producing roots (67%), as compared to the other IBA concentrations. Generally, the roots were short (less than 20 mm), but fibrous. There was no significant difference between 0.5 and 0.75 mg l^{-1} IBA in the percentage of shoots producing roots. The medium supplemented with 0.75 mg l^{-1} IBA did however, produce roots of a better quality. These roots were longer and also fibrous. Shoots cultured on MS lacking plant growth hormones also produced some roots (one root per shoot) (Figure 8C). However, hyperhydricity was often a problem. Symptoms were observed on the shoots.

3.3.1.3.1 Problems with hyperhydricity

While medium containing NAA also initiated roots they were not of the same quality, being short and stubby, with swelling at the base of the stem. Callusing at the base of the shoots was observed. This is a problem, which directly inhibits shoot growth and further development. This in turn affects rooting greatly (McCOWN and McCOWN 1987). The leaves of these plants also appeared to be glassy and translucent (Figure 8D), a morphological effect characterising hyperhydric plants (DEBERGH, HARBAOUI and LEMEUR 1981). When compared with normal leaves of an *in vitro* grown *C. paniculata* plantlet, the hyperhydric leaves had a glaucous green colour, were narrow and curled. When hyperhydric *C. paniculata* plantlets

described above were planted *ex vitro*, there was no establishment. This scenario is usually experienced when acclimatizing hyperhydric plants. It is usually possible to avoid hyperhydricity and reverse it if the phenomenon is not too advanced (PÂQUES 1991).

Replacing Gelrite with agar or increasing the gel strength helps to reduce hyperhydricity (DEBERGH, HARBAOUI and LEMEUR 1981; ZIV, MEIR and HALEVY 1983; VON ARNOLD and ERIKSSON 1984; BURNS, SUTTER and WOZNIAK 1996). There is a strong connection between the culture medium hardness, the proliferation rate and hyperhydricity. Lowering the nutrient gel content leads to an increase in both proliferation and hyperhydricity rate. Therefore the high proliferation rate needs to be sacrificed to obtain good quality plantlets by increasing the medium hardness (ZIV, MEIR and HALEVY 1983). After 35 days on MS without growth hormones, there was no swelling at the base of the stem in all the plantlets. Roots of better quality were observed. They were white and fibrous and not 'fat' as before (Figure 8E). All the hyperhydric shoots transferred to MS solidified with 8 gl^{-1} were recovered. They regained their normal colour and the glassy appearance disappeared. No swelling or callus was observed at the bases of the stems. These plants were prepared for acclimatization.

3.3.1.4 Acclimatization

Gelrite or agar (in the case of shoots that were transferred to this medium because of hyperhydricity) was carefully washed off the cultured plantlets. The plantlets were planted on a mixture of potting soil and vermiculite (1:1)(v/v). Problems were often encountered when the gelling agent

attached to the roots has to be removed and the roots transferred to soil. Plantlets in the mist house started to produce new leaves after 3 days. After 21 days, the leaves assumed a dark green colour. These plants were transferred to a green house (23±2°C) (Figure 8F) and the survival rate after 35 days was 63%.

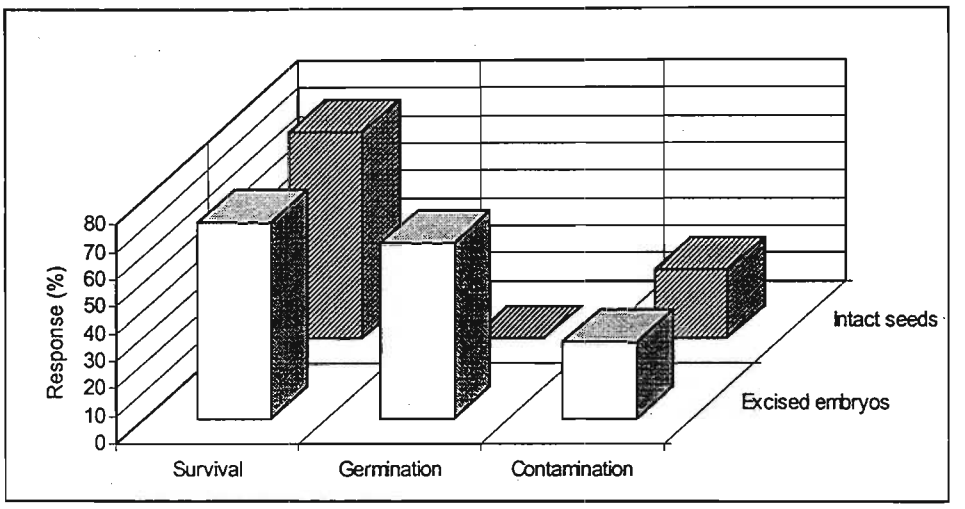


Figure 7. Response of excised embryos and intact seeds of *C. paniculata* after surface decontamination.

Figure 8. *In vitro* response of *C. paniculata*. (A) *In vitro* germinated *C. paniculata* seedling. (B) Shoots regenerated from nodal explants cultured on medium containing 2, 2.5 and 3 mg l^{-1} BA (from left to right). (C) Roots formed on a shoot subcultured to medium containing 0.75 mg l^{-1} IBA. (D) Roots formed on shoots subcultured to MS medium without growth hormones. (E) Hyperhydric shoots on medium solidified with 3 g l^{-1} gelrite, there is callus at the base of the shoot. (F) Roots formed after a shoot was subcultured from MS medium supplemented with 1 mg l^{-1} NAA and solidified with gelrite to agar-solidified medium without growth hormones. (G) Acclimatized plantlets after 35 days.

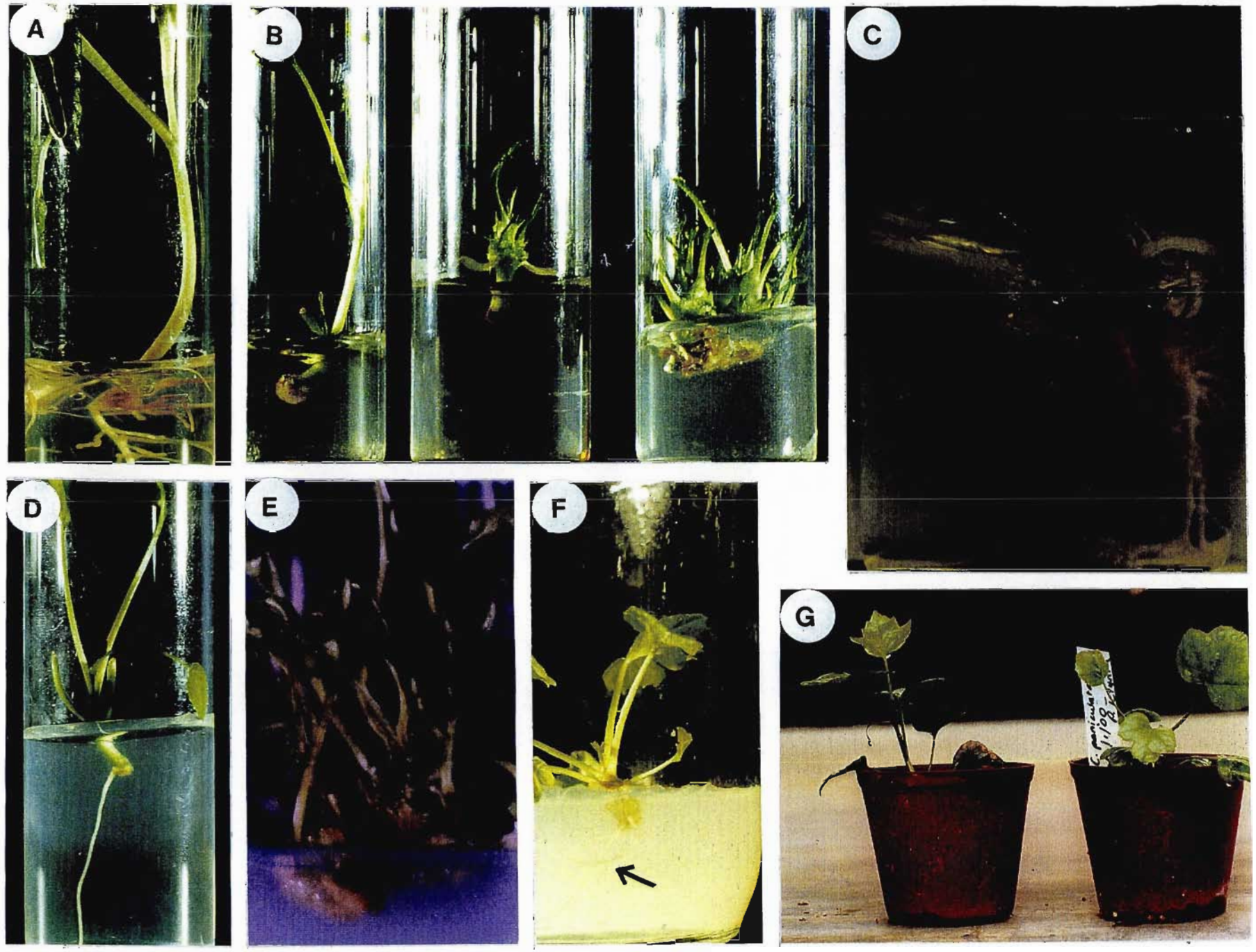
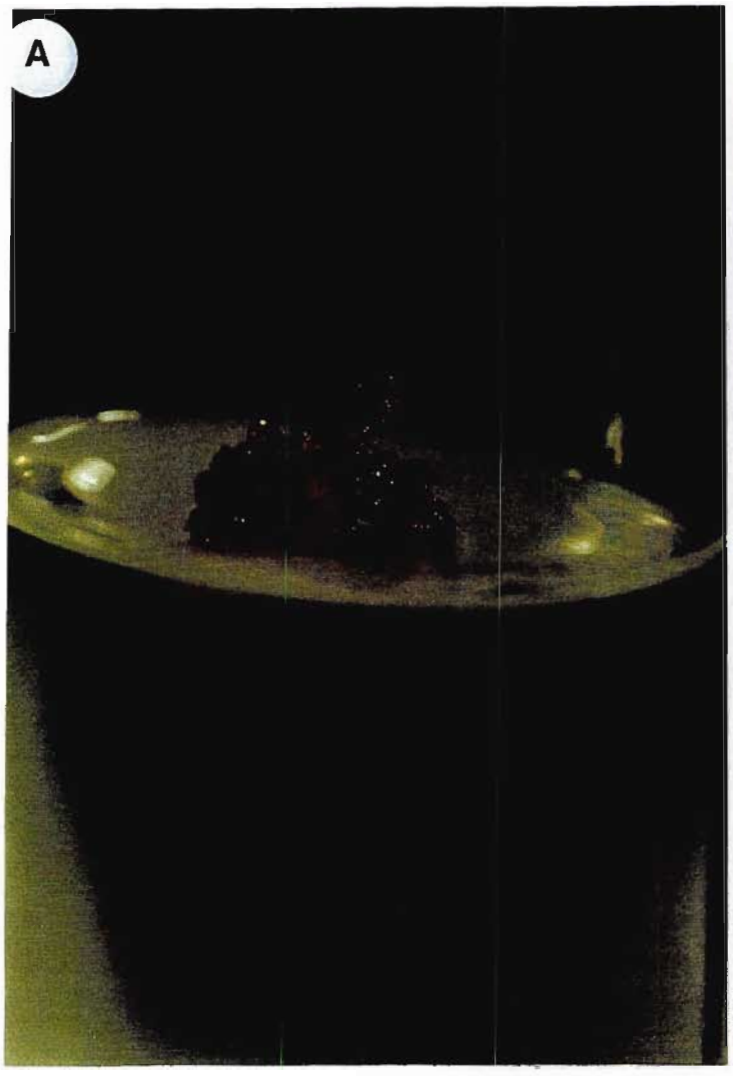
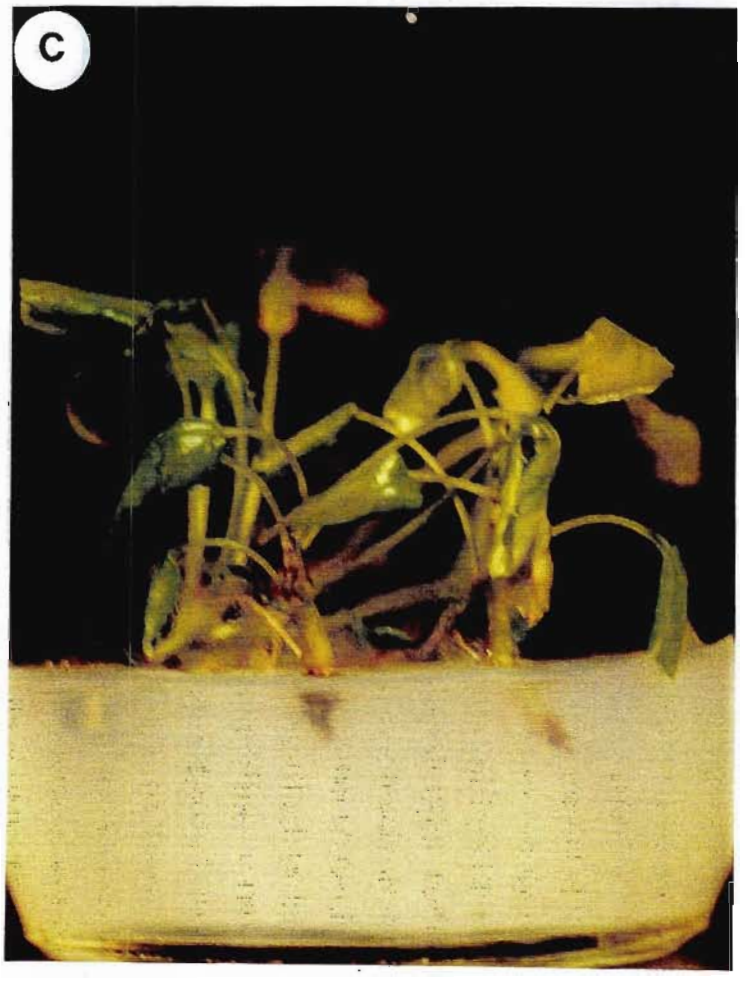

A
B
C
D
E
F
G

Figure 9. *In vitro* response of *C. paniculata* zygotic embryos on MS supplemented with a combination of 2,4-D and BA. (A) Callus formed when zygotic embryos were cultured on 1.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA. (B) Plantlet regeneration after subculturing to MS medium without plant growth regulators. (C) Separated shoots subcultured to rooting medium supplemented with 0.75 mg l^{-1} IBA showing normal *in vitro* growth before roots were observed.


A


B


C

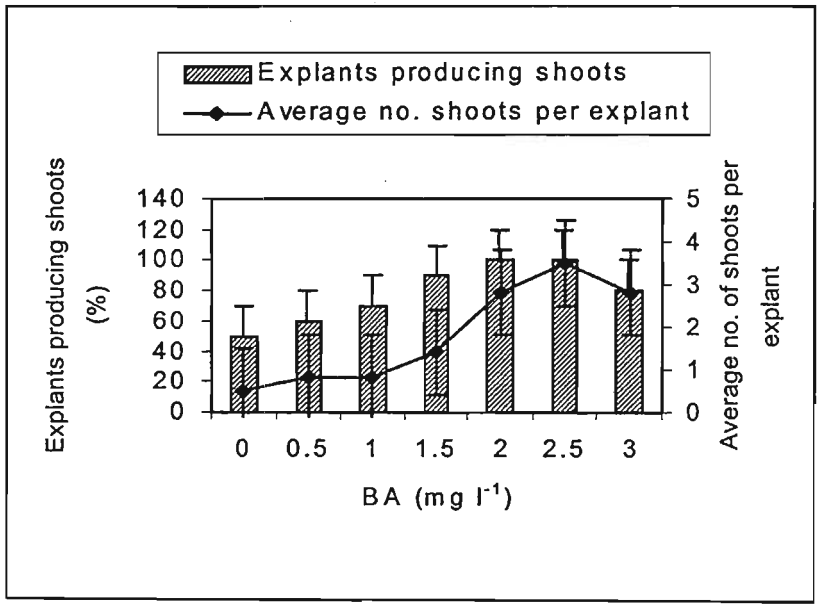


Figure 10. Effect of various concentrations of BA on the percentage of *C. paniculata* nodal explants producing shoots and the number of shoots produced per explant.

Table 3.1 *Effect of various concentrations of BA and 2,4-D on zygotic embryo growth of* C. paniculata

	2,4-D (mg l^{-1})					
BA (mg l^{-1})	1	1.5	2	2.5	3	3.5
0.5	NC	C	C	C	C	C
1	NC	NC	C	NC	C	NC
2	NC	NC	NC	NC	C	NC

C = Callus observed, NC = No callus observed

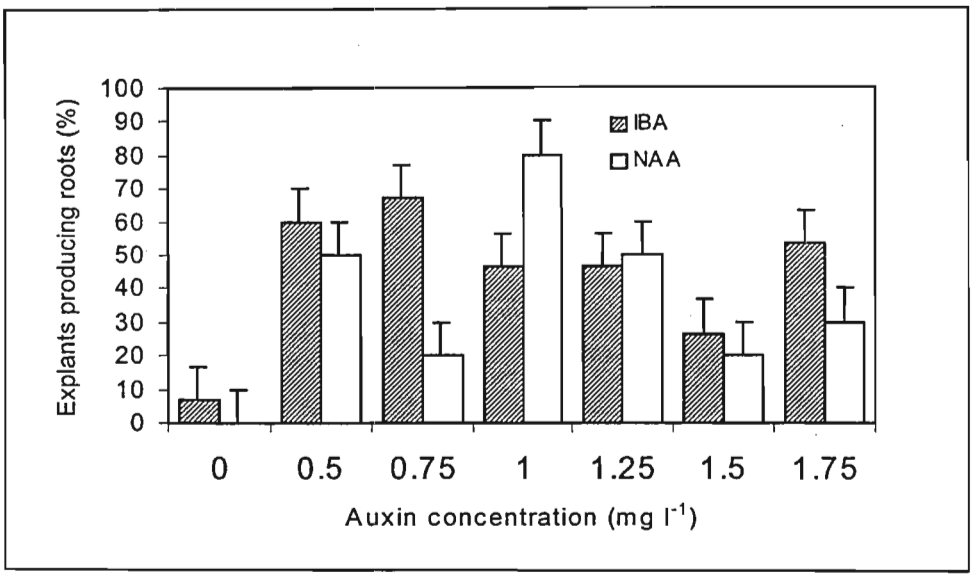


Figure 11. Effect of various auxin concentrations (mg l^{-1}) on the percentage of *C. paniculata* shoots producing roots.

3.3.2 Studies on *C. spicata*

3.3.2.1 Decontamination of plant material

The first trial of decontamination was not very successful. The highest percentage of survived explants after a period of 14 days was less than 50%. The explants in the first trial were treated with 0.01% $HgCl_2$, excluding the modifications of trial 2. Browning of explants was observed after the first surface decontamination. There was a distinct improvement in the quality of explants after the second trial. This was due to the introduction of the ascorbic acid soaking step at the end of the decontaminating procedure. In the first trial 80% of the explants showed browning while in the second trial no browning was observed. Browning is known to be indicative of the oxidation of polyphenolic compounds inside the culture vessel (DIRR and HEUSER 1987, BONGA AND VON ADERKAS, 1992). Explants that become discoloured by oxidative browning often do not establish in culture (DIRR and HEUSER 1987). Therefore, ascorbic acid functioned as an effective antioxidant in reducing the browning.

In subsequent experiments, explants were decontaminated with a treatment with 70% ethanol, soaking in 0.2% Benlate, treatment with 0.01% $HgCl_2$ and soaking in 50 mg l^{-1} ascorbic acid. The success of this treatment ranged from 50-80% in terms of decontaminated explants (Figure 12). Generally, there was an increase in the number of contamination-free explants with an increase in the time of treatment. The surface decontamination of shoot-tips was not laborious. However, it is recommended that shoot-tips do not be over-exposed to the decontamination agent.

Over-exposure led to explant damage by $HgCl_2$ during surface decontamination. Usually, the innermost meristem tip is free of contamination. Therefore, surface decontamination is mainly targeted to remove surface microbes. The success of decontamination is also influenced by excision. Because the shoot-tips were tiny, there was often damage of the explant due to excision. When explants are exposed to the decontamination agent, it penetrates them and the explants are killed by it.

Most of the contamination observed was fungal. Ideally, the spraying of the plants prior to surface decontamination is designed to eliminate most of the contaminants and to obtain good quality stock plants. Since the plants were irrigated by an automated irrigating system, sometimes the fungicide was washed off before absorption. Therefore, Stage 0 of *in vitro* multiplication must be given more attention. This stage is important because it ensures healthier and uniform stock plants (MURASHIGE 1974, DEBERGH and MAENE 1981).

3.3.2.2 Explant source and size

All explants were derived from *C. spicata* trees. The shoot apices (0.4 cm) with a few leaf primodia were cultured onto the specified medium. It was often difficult to obtain more than one shoot-tip from a single plant. Chiefly, *Cussonia* species confer strong apical dominance therefore, they do not have more than one shoot-tip. The shoot-tips do re-sprout after a while. It usually takes between one and three months for the plants to produce new growing tips. The re-sprouting of shoot-tips compensates for destructive harvesting when shoot-tips are excised.

Although shoot-tips used in this project were slightly larger than ideal apical meristems, it was often difficult working with them. Explant size influences the rate and success of regeneration. Small explants are difficult to excise and manipulate. Thus, they become damaged during excision or become desiccated while manipulating them. Ideally, it is desirable to use very small apical meristems for production of disease-free plantlets. However, apical meristems have a very low survival rate. Therefore, they are impractical for rapid propagation purposes (DODDS and ROBERTS 1985).

3.3.2.3 Media and supplements

The limited literature with respect to tissue culture studies on *Cussonia* species led to the use of MS medium as the basal medium in the experiments. MS medium has been used for years and is still used in a wide range of experiments involving plant regeneration from shoot-tips. The presence or absence of plant growth regulators influenced the regeneration of explants.

A regeneration percentage of less than 20% was observed with shoot-tip explants cultured on MS supplemented with a combination of BA and NAA. A regeneration of 50% was observed from explants cultured on MS with 2 mg l^{-1} BA and 0.5 or 1 mg l^{-1} IBA (Figure 13A). This was the highest regeneration percentage obtained when BA was used in combination with IBA.

Explants that produced shoots were also observed in cultures maintained on MS medium with 2 mg l^{-1} BA and 1 mg l^{-1} IAA. The regeneration percentage was between 60 and 80% (Figure 13B). Combinations of

BA and IAA proved to be better for plantlet production than combinations of BA and NAA or BA and IBA. With BA and IAA regeneration was up to 80% while in the other combinations it was below 50%. No abnormalities were observed in the shoots produced with all combinations of auxin and cytokinin.

It must be noted that in shoot-tip culture of *C. spicata*, no multiple shooting was observed. A single explant produced only one shoot with a flush of leaves. The shoots grew up to 4.5±2 cm in height (Figure 14A and B). Considering occurrence in the natural environment, when mature *Cussonia* trees grow, only one new flush of leaves is produced in the new season at the growing point of each branch (STREY 1973). This phenomenon was also observed when shoot-tips isolated from mature trees of *C. spicata* were placed in culture. No rooting was observed on the shoots produced, therefore no acclimatization was attempted.

The protocol described in this study may form the basis of further research on germplasm conservation and *in vitro* propagation of *Cussonia* species. With further development of the protocol, *Cussonia* species could be economically propagated either for ornamental or medicinal purposes. Emphasis could be on improving multiple shooting and rooting experiments. Since acclimatization has already been developed for *C. paniculata* species, transferring *in vitro* propagated plantlets to the natural environment would apparently be relatively easy.

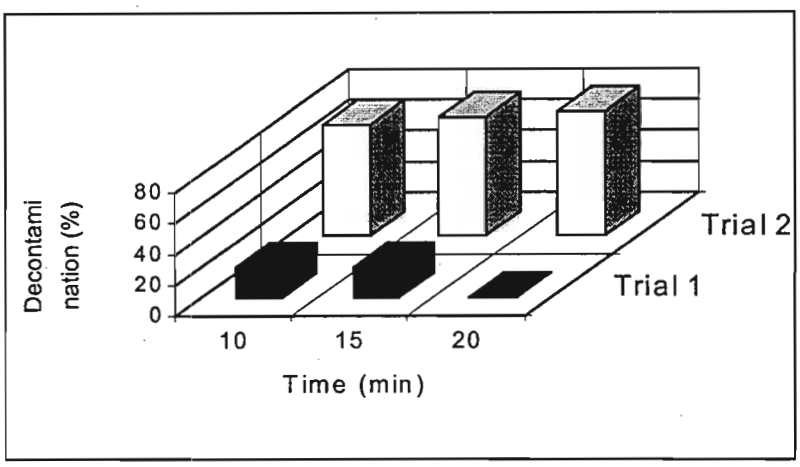


Figure 12. Effect of duration and decontamination procedure on the percentage of aseptic *C. spicata* shoot-tips when using 0.01% $HgCl_2$.

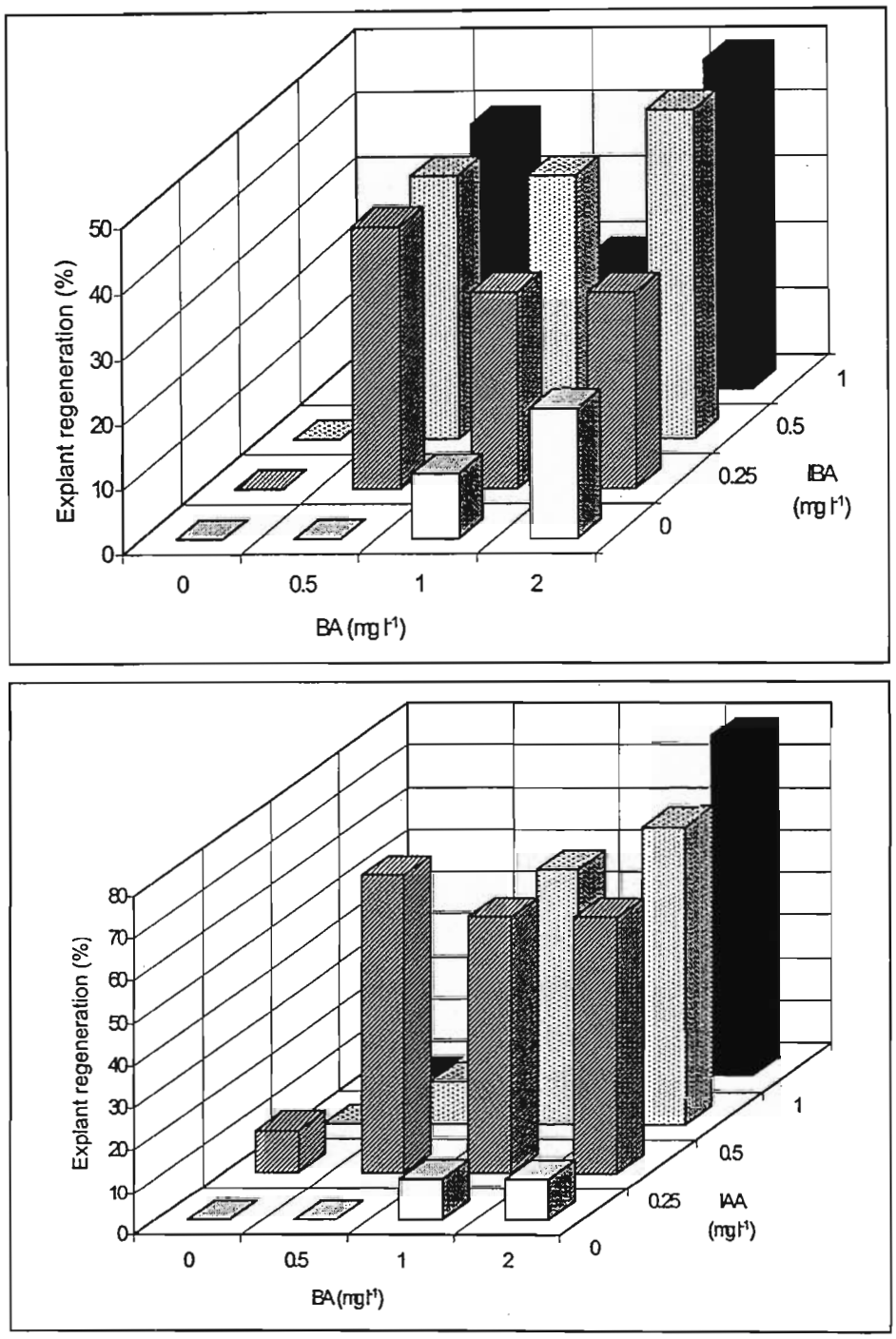
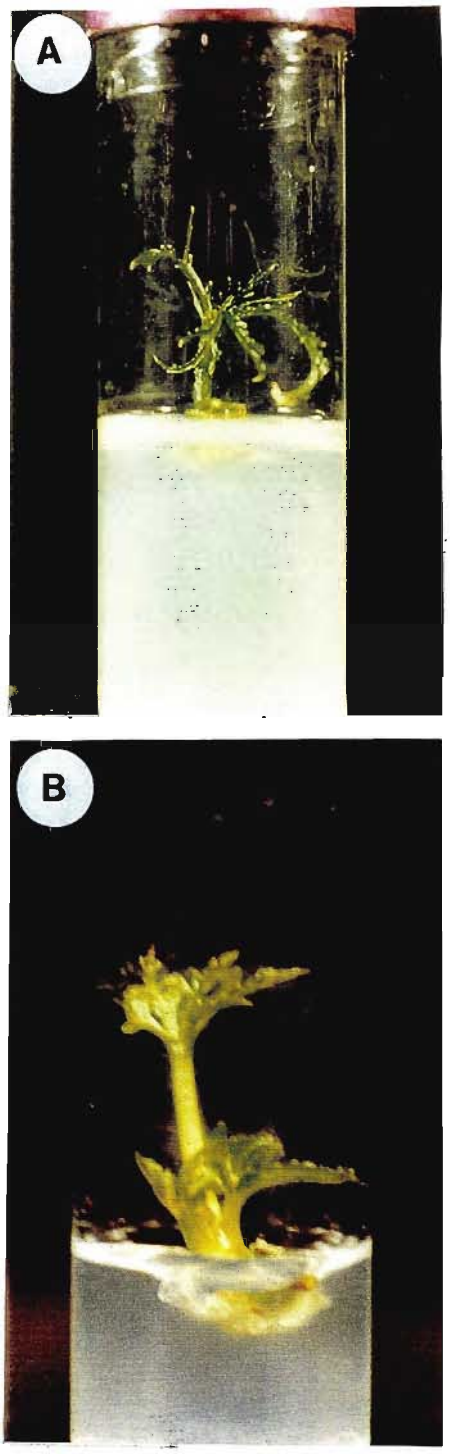


Figure 13. Effect of various combinations of BA and IBA (A), and BA and IAA (B) on the percentage *C. spicata* shoot-tip explants producing shoots.

Figure 14. Shoots regenerated from *C. spicata* shoot tips cultured on MS with 0.5 mg l^{-1} IBA and 1 mg l^{-1} BA (A) and 1 mg l^{-1} IAA and 2 mg l^{-1} BA (B).


A
B

CHAPTER 4

GENERAL CONCLUSIONS

Cussonia species (commonly known as Cabbage trees) are indigenous to South Africa and are used in traditional medicine to treat an assortment of diseases. Due to their attractive growth form, they are assets in gardens. However, there are no developed methods for propagating these species. In the previous Chapters, the use of three selected species, *Cussonia paniculata* (Eckl. & Zeyh.), *C. spicata* (Thunb.) and *Schefflera umbellifera* (Sond.) Baill. (= *C. umbellifera*), in traditional medicine was validated. Rapid propagation protocols for *C. paniculata* and *C. spicata* were investigated and ultimately developed for the former species.

Cussonia paniculata, *C. spicata* and *C. umbellifera* were screened for their medicinal properties, mainly focussing on anti-bacterial, anti-inflammatory and anti-malarial activities. In the anti-bacterial screening, *C. spicata* bark and root extracts showed activity against selected Gram-positive and Gram-negative bacterial strains at a concentration of 50 mg ml^{-1}. The highest inhibition was observed with ethanol and ethyl acetate root extracts against *Staphylococcus aureus*. The other two species did not show anti-bacterial activity. Ethanol and ethyl acetate extracts of all species showed anti-inflammatory activity at a concentration of 8 µg ml^{-1}. These active extracts showed an inhibition percentage that was greater than 50 % against cyclooxygenase. All three species are used in traditional medicine to treat diseases associated with inflammation. In the anti-malarial screening, bark extracts were screened. *C. umbellifera* bark extracts exhibited the best inhibition against *P. falciparum*, a malaria-causing agent in humans. The percentage inhibition of these extracts was up to 100% at a concentration of 200 µg ml^{-1}. While *C. spicata* is known

to be used to treat malaria, the screening results showed much less activity (less than or equal to 35 %) as compared to *C. umbellifera*, which is preferably used to treat malaria. The results obtained from screening these three species validated their use in traditional medicine. Meaning that the people or traditional healers use these species for different treatments by possibly relying on past knowledge about the effects after administering the medicine. For example, they use *C. spicata* to treat infections and other venereal diseases. When this species was screened for medicinal properties, it showed anti-bacterial activity. Therefore the use of *C. spicata* in traditional medicine to treat infections is validated because the screening indicates that it has anti-bacterial compounds. Fingerprinting using Thin Layer Chromatography (TLC) was used in an attempt to determine whether there are any chemical differences or similarities between the three species. There were similarities between the plant parts across the species as well as some differences. However, this method cannot be used as an unequivocal test to deduce that compounds that are present in a certain species and not in others are the ones responsible for bringing about a certain biological activity. That can only be achieved by a bioassay-guided isolation of compounds.

A tissue culture protocol was developed to produce a large number of plants of *C. paniculata*. Explants were derived from nodal explants of *in vitro* germinated seeds and cultured on Murashige and Skoog (MS) (1962) medium supplemented with 3% sucrose, 2.5 mg l^{-1} BA and solidified with 3 g l^{-1} Gelrite,. These explants produced multiple shoots. The average number of shoots per explant ranged between 1 to 3.5. Multishoots were subcultured on to rooting media and roots were produced on MS with 0.75 mg l^{-1} IBA and 1 mg l^{-1} NAA. Callus from zygotic embryos also produced plantlets on MS supplemented with 1.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA. Hyperhydricity was encountered in this study. This problem was reversed successfully by transferring the shoot from

medium solidified with 3 g l^{-1} Gelrite to medium solidified with 8 g l^{-1} agar. Plantlets were successfully acclimatized for planting *ex vitro*.

C. spicata was not successfully micropropagated from shoot-tip explants. However, a protocol was developed for decontaminating shoot-tips from the mother plants. A high percentage of shoot-tip regeneration was observed when they were cultured on MS medium supplemented with 2 mg l^{-1} BA, 1 mg l^{-1} IAA and 1 mg l^{-1} GA_3. However, multishoots were not observed as in *C. panicualata*. Shoot elongation *in vitro* was similar to shoot elongation as it occurs in nature. The shoots elongated and a flush of palmitately arranged leaves were produced. When *Cussonia* trees grow, a new flush of leaves is produced at the growing point with each new season. This study could therefore be a basis for further development of a commercially viable protocol for rapid propagation and conservation of the germplasm of *Cussonia* species.

REFERENCES

AKERELE, O. (1991) The conservation of medicinal plants: Proceedings of an international Consultation 21-27 March 1988 held at Chiang Mai, Thailand. O. Akerele, V. Heywood and H. Synge (eds). Cambridge University Press, Cambridge.

BALANDRIN, M. F., A. D. KINGHORN and N. R. FARNSWORTH (1993) Plant-Derived Natural Products in Drug Discovery and Development: An Overview. In: Human medicinal agents from plants. A. D. Kinghorn and M. F. Balandrin (eds). American Chemical Society Symposium Series 534, Washington. pp 1-10.

BHOJWANI, S. S. and M. K. RAZDAN (1983) Developments in crop science. In: Plant tissue culture: theory and practise (Vol. 5) Elsevier, Amsterdam.

BIONDI, S. and T. THORPE (1982) Growth regulator effects, metabolite changes, and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. ***Botanical Gazette*** 143: 15-20.

BONGA, J. M. (1982) Vegetative propagation in relation to juvenility, maturity and rejuvenation. In: Tissue Culture in Forestry. J. M. Bonga and D. J. Durzan (eds). The Hague: Martinus Nijhoff/Junk. pp 387-412.

BONGA, J. M. and P. VON ADERKAS (1992) *In vitro* culture of trees. Kluwer Academic publishers, Netherlands.

BRADBEER, J. W. (1998) Seed dormancy and germination. Chapman and Hall, New York. pp 38-55.

BRAINERD, K. E., L. H. FUCHIGAMI, S. KWIATSKOWKI and C. S. CLARK (1981) Leaf anatomy and water stress of aseptically cultured 'Pixy' plum grown under different environments. ***Horticultural Science*** 16: 173-175.

BURNS, J. A., E. SUTTER, J. GRIFFIS and C. A. WOZNIAK (1996) Internet Discussion: Gelling Agents and Hyperhydricity (Vitrification) ***Agricell Report***, September, pp 19.

BURROWS, G. E., D. D. DOLEY, R. J. HAINES and D. G. NICKLES (1988) *In vitro* propagation of *Auraucaria cunninghamii* and other species of the Auraucariaceae via axillary meristems. ***Australian Journal of Botany*** 36: 665-676.

CHOI, K. T., J. C. PARK and I. O. AHU (1990) Saponin production in tissue culture of ginseng. ***Korean Journal of Ginseng Science*** 14:2, 107-111.

COATES PALGRAVE, K. C., M. COATES PALGRAVE and P. COATES PALGRAVE (1985) Everyone's guide to Trees of South Africa. Central News Agency, Johannesburg.

CORBINEAN, F., F. ENGELMANN and D. CÔME (1990) Ethylene production as an indicator of chilling in oil palm (*Elaeis guineensis* Jacq.) somatic embryos. ***Plant Science*** 71: 29-34.

CURREL, B. R., J. W. JAMES, C. K. LEACH, R. A. PATMORE, M.C. E. VAN DAM-MIERAS, W. H. DE JEU, J. DE VRIES (The Biol team) (1993) *In Vitro* Cultivation of Plant Cells. Butterworth-Heinemann Ltd. University of Greenwich (United Kingdom) and Open Universiteit, The Netherlands.

DEBERGH, P., J. AITKEN-CHRISTIE, D. COHEN, B. GROUT, S. VON ARNOLD, R. ZIMMERMAN and M. ZIV (1992) Reconsidering of the term 'vitrification' as used in micropropagation. ***Plant Cell, Tissue and Organ Culture*** 30: 135-140.

DEBERGH, P., Y. HARBAOUI and R. LEMEUR (1981) Mass propagation of globe artichoke (*Cynara scolymus*): evaluation of different hypotheses to overcome vitrification wit special reference to water potential. ***Physiologia Plantae*** 53: 181-187.

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

DING, J. Y., Q. CHEN, D. J. XIANG, X. HE (1993) Studies on medicinal products from *Panax ginseng* cell culture. In: Biotechnology in agriculture. C. B. YOU and Z. L. CHEN (eds). Proceedings of the First Asia-Pacific Conference on Agricultural Biotechnology, Beijing, China, 20-24 August 1992. Current Plant Sicence and Biotechnology in Agriculture 15: 291-295. Kluwer Academic Publishers, Dordrecht.

DIRR, M. A. and C. W. HEUSER, Jr (1987) The Reference Manual of Woody Plant Propagation: From Seed to Tissue Culture. Varsity Press, Inc., Athens.

DODDS, J. H. and L. W. ROBERTS (1985) Experiments in Plant Tissue Culture (2nd Ed.). Cambridge University Press, Cambridge. pp 113-121.

DUBOIS, M. A., M. ILYAS and H. WAGNER (1986) Cussonosides A and B, two Triterpene-saponins from *Cussonia barteri*. ***Planta-Medica*** 2: 80-83.

EARLE, E. D. and R. W. LANGHANS (1975) Carnation propagation from shoot tips cultured in liquid medium. ***Horticultural Science*** 10: 608-610.

FINNIE, J. F. (1988) Tissue Culture of Selected Indigenous Monocotyledons, PhD thesis. University Press, University of Natal, Pietermaritzburg.

FLINN, B. S., D. T. WEBB and W. GEORGIS (1986) *In vitro* control of caulogenesis by growth regulators and media components in embryonic explants of Eastern white pine (*Pinus strobus*). ***Canadian Journal of Botany*** 64: 1948-1956.

FOURIE, T. G., E. MATTHEE and F. O. SNYCKERS (1989) A pentacyclic triterpene acid, with anti-ulcer properties, from *Cussonia natalensis* ***Phytochemistry*** 28: 10, 2851-2852.

GAMBORG, O. L., R. A. MILLER and K. OJIMA (1968) Nutrient requirements of suspension cultures of soybean root cells. ***Experimental Cell Research*** 50: 151-158.

GENTRY, A. H. (1993) Tropical Forest Biodiversity and the Potential for New Medicinal Plants. In: Human medicinal agents from plants. A. D. Kinghorn and M. F. Balandrin (eds). American Chemical Society Symposium Series 534, Washington.

GEORGE, E. F., D. J. M. PUTTOCK and H. J. GEORGE (1987) Plant Culture Media, Vol. 2, Exegetics Ltd, Edington.

GROUT, B. W. W. and H. ASTON (1977) Transplanting of cauliflower plants regenerated from meristem culture 1. Water loss and transfer related to changes in leaf wax and to xylem regeneration. ***Horticultural Research*** 17: 1-7.

GUMEDE, M. V. (1989) Traditional Healers - a Medical Doctor's Perspective. Skotaville, Johannesburgh.

GUNZINGER, J., J. D. MSONTHI and K. HOSTETTMANN (1986) Molluscicidal saponins from *Cussonia spicata*. ***Phytochemistry*** 25: 11, 2501-2503.

HABERLANDT, G. (1902) Kultinversuche nit isolierten pflanzellen, Sitzungsberichte der Akademie der Nissenschaften in Wien, Mathematisch-Naturwissen-schaftiche Wasse 111: 69-92.

HARRY, I. S. and T. A. THORPE (1994) *In vitro* culture of forest trees. In: Plant cell and tissue culture. I. K. Vasil and T. A. Thorpe (eds). Kluwer Academic Publishers, London.

HOPPE, H. C. (1993) Identification and characterization of selected merozoite-stage antigens in southern Africa isolates of *Plasmodium falciparum*. PhD thesis, University of Pretoria, Pretoria.

HUSSEY, G. (1980) *In vitro* propagation. In: Tissue culture methods for Plant Pathologists. D. S. Ingram and J. P. Helgeson (eds). Blackwell, Oxford. pp 51-62.

HUTCHINGS, A., A. H. SCOTT, G. LEWIS and A. B. CUNNINGHAM (1996) Zulu medicinal plants: an inventory. University of Natal Press, Pietermaritzburg.

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

JOHNSON, D. and S. JOHNSON (1993) Gardening with indigenous trees and shrubs. Southern Book Publishers, Cape Town.

LEOPOLD, A. C. (1987) Contemplations on hormones as biological regulators. In: Hormone action in Plant Development: A critical Appraisal. G. V. Hoad, J. R. Lenton, M. B. Jackson, and R. K. Atkin (eds). Butterworths, London. pp 3-5.

LITVAY, J. D., M. A. JOHNSON, D. VERMA, D. EISPAHR and K. WEYRAUCH (1981) Conifer suspension culture medium development using analytical data from developing needs. IPC Technical Paper Series, No. 115.

LLYOD G. and B. McCOWN (1980) Commercially-feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. ***International Plant Propagation Society Proceedings*** 30: 421-427.

LOO, S. (1945) Cultivation of excised stem tips of Asparagus *in vitro*. ***American Journal of Botany*** 32: 13-17.

MAKLER, M. T., J. M. RIES, J. A. WILLIAMS, J. E. BANCROFT, R. C. PIPER, B. L. GIBBINS and D. J. HINRICHS (1993) Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. ***American Journal of Tropical Medicine and Hygiene*** 48 (6): 739.

MATSUTA, N. and T. HIRABAYASHI (1989) Embryogenesis cell lines from somatic embryos of grape (*Vitis vinifera* L.). ***Plant Cell Reports*** 7: 684-687.

McCOWN, D. D. and B. H. McCOWN (1987) North American Hardwoods. In: Cell and Tissue Culture in Forestry, Vol. 3, Case Histories: Gymnosperms, Angiosperms and Palms. J. M. Bonga and D. J. Durzan (eds). Martinus Nijhoff Publishers, Dordrecht, pp 247-260.

MONTEUUIS, O. (1987) *In vitro* meristem culture of juvenile and mature *Sequiadendron giganteum*. ***Tree Physiology*** 3:265-272

MUIR, W. H. (1953) Cultural Conditions Favouring the Isolation and Growth of Single Cells from Higher Plants *In Vitro*. PhD. Thesis, University of Wisconsin, United States of America.

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

MURASHIGE, T. (1974b) Plant propagation through tissue cultures. ***Horticultural Science***, 9 (3): 2-3.

MURASHIGE, T. (1976) Clonal crops through tissue culture. In: Plant tissue culture and its bio-technological application. W. Barz, E. Reinhard and M. H. Zenk (eds). Springler Verlag, Berlin, Heidelberg, New York, pp 392-403.

MURASHIGE, T. (1978) Principles of rapid propagation. In: Propagation of higher plants through tissue culture. A bridge between research and application. K. W. Hughes, R. Henke and M. Constantin (eds). Technical information centre, United States Department of Energy. Springfield, Virginia. pp 14-24.

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

NISSEN, S. J. and E. G. SUTTER (1988) Stability of IAA and IBA in nutrient medium after autoclaving and after storage under various environmental conditions. ***Horticultural Science*** 23: 758-761.

PALMER, E. and N. PITMAN (1961) Trees of South Africa. Balkema, Cape Town.

PAPAJEWSKI, S., J. -H. GUSE, I. KLAIBER, G. ROOS, R. SÜßMUTH, B. VOGLER, C. U. WALTER and W. KRAUS (1998) Bioassay guided isolation of a new C18-polyacetylene from *Cussonia barteri*. ***Planta-Medica*** 64: 5, 479-481.

PÂQUES, M. (1991) Vitrification and Micropropagation: causes, remedies and prospects. ***Acta Horticulturae*** 289: 273-282.

PÂQUES, M. and P. BOXUS (1987) A model to learn 'vitrification', the rootstock apple M.26. Present results. ***Acta Horticulturae*** 212: 193-210.

PICK, W. M. (1992) Primary Health care - some lessons for South Africa. ***South African Medical Journal*** 82: 300-301.

QUAK, F. (1977) Meristem culture and virus-free plants. In: Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. J. Reinert and Y. P. S. Bajaj (eds). Berlin-Heidelberg, New York. pp 598-615.

RASOANAIVO, P. and S. RATSIMAMANGA-URVERGA (1993) Biological Evolution of plants with reference to malagasy flora. Monograph for the IFS-NAPRECA Workshop on Bioassays, Antanaivo, Madagascar.

SCHWANN, T. (1839) Microskopische Untersuchungen über die Übereinstimmung in der Struktur und dem Wachstume die Tiere und Pflanzen. No. 176, Oswalds Klassiker der exakten Wissenschafte. W. Engelman, Leipzig.1910.

SAKAMOTO, K., K. SAWAMURA, K. IIDA, K. HAJIRO, Y. ASADA, T. YOSHIKAWA and T. FURUYA (1993) Effects of nutrients on anthocyanin production in cultured cells of *Aralia cordata*. ***Phytochemistry*** 33: 2, 357-360.

SKOOG, F. and C. O. MILLER (1957) Chemical regulation of growth and organ formation in plant tissue grown *in vitro*. ***Symposia of the Society of Experimental Biology*** 11: 118-131.

STREY, R. G. (1973) Notes on the genus *Cussonia* in South Africa. ***Bothalia*** 11: 191-201.

TRAGER, W. and J. B. JENSEN (1976) Human malaria parasites in continuous culture. ***Science*** 19: 4254, 673-675.

VAN WYK, B. E. and N. GERICKE (2000) People's plants: A guide to Useful Plants of southern Africa. Briza Publications, Pretoria.

VENTER, F. and J-A. VENTER (1996) Making the most of indigenous trees. Briza Publications, Pretoria.

VON ARNOLD, S. and T. ERIKSSON (1984) Effect of agar concentration on growth and anatomy of adventitious shoots of *Picea abies* (L.) Karst. ***Plant Cell, Tissue and Organ Culture*** 3: 257-264.

WAANE, S. A. C. (1990) The use of traditional medicinal plants: the cultural content. Proceedings of an International Conference of Experts from

Developing countries on Traditional Medicinal Plants. University of Tanzania Press, Dar es Salaam.

WAGNER, H. and S. BLADT (1996) Plant drug analysis: A Thin Layer Chromatography Atlas (2nd Ed.). Springler-Verlag, Berlin, Heidelberg, New York.

WALKER, C. C. (1988) *Cussonia paniculata*: the mountain Cabbage tree. ***British Cactus and Succulent Journal*** 6: 98-100.

WATT, J. M. and M. G. BREYER-BRANDWIJK (1962) The medicinal and poisonous plants of Southern Africa and Eastern Africa (2nd Ed.). Livingstone, Edingburg.

WEBB, K. J. and H. E. STREET (1977) Morphogenesis *in vitro* of *Pinus* and *Picea*. ***Acta Horticulturae*** 78: 29-34.

WEEKS, J. R. (1973) The Prostaglandins: Their Nature, Formation ad General Pharmacology. In: Prostaglandins and cyclic AMP: Biological Actions and Clinical Applications. R. H. Kahn and W. E. Lands (eds). Academic Press, New York. pp 1-15.

WHITE, P. R. (1943) A handbook of plant tissue culture. Jaques Cattel press, Lancaster, Pa.

WHITE, H. L. and A. T. GLASSMAN (1974) A simple radiochemical assay for prostaglandin synthetase. ***Prostaglandins*** 7: 123-129.

ZIV, M., G. MEIR and A. H. HALEVY (1983) Factors influencing the production of hardened glacous carnation plantlets *in vitro*. ***Plant Cell, Tissue and Organ culture*** 2: 55-60.