Micropropagation and acclimatization of *Aloe polyphylla* and *Platycerium bifurcatum*

BY

JUDE CHINEDU CHUKWUJEKWU

submitted in fulfilment of the requirements for the degree of
(Master of science)

in the Research Centre for Plant Growth and Development, School of Botany and Zoology, Faculty of Science and Agriculture.
University of Natal, Pietermaritzburg

June, 2001

PREFACE

With great pleasure, I wish to declare that this thesis, submitted for the degree of Master of Science in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg, except where the work of others is acknowledged, is the result of my own investigation.

.....
JUDE CHINEDU CHUKWUJEKWU
JUNE 2001

We certify that the above statement is correct.

.....
PROFESSOR J VAN STADEN
(SUPERVISOR)

.....
MISS C W FENNELL
(CO-SUPERVISOR)

PUBLICATIONS FROM THIS THESIS

CHUKWUJEKWU JC, FENNELL CW and VAN STADEN J (2001) Micropropagation and acclimatization of *Aloe polyphylla* (In preparation).

CONFERENCE CONTRIBUTIONS FROM THIS THESIS

CHUKWUJEKWU JC, FENNELL CW and VAN STADEN J (2000) Micropropagation and acclimatization of *Aloe polyphylla* (paper). Second Annual Research Centre Meeting, University of Natal, Pietermaritzburg.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Professor J. Van Staden (supervisor) and Miss C W Fennell (co-supervisor) for their guidance during this investigation and in the preparation of this manuscript.

To Dr. Mia Abrie, thank you for taking me through tissue culture for the first time. It was really great.

To my parents and Dr. C I Chukwujekwu, thank you for your encouragement and financial support throughout my years of study.

To every member of the Research Centre for Plant Growth and Development, especially Cathy Ford, Georgina Arthur and Noxwenda Makunga, for your ever available assistance. You were all a source of inspiration to me.

Lastly, but not the least, to Almighty God, whom by his grace made it possible for me to successfully complete this work. I give you all the glory and honour.

ABSTRACT

Shoot cultures of *Aloe polyphylla* were initiated from young shoot explants of *in vitro* grown plants. The basal medium was MS medium (MURASHIGE and SKOOG, 1962), supplemented with 100 mgl^{-1} myo-inositol, and 30 gl^{-1} sucrose. Agar (0.8 %) was used as the gelling agent. Different cytokinins, singly or in combination with auxins (IBA and NAA), were tested for shoot proliferation activity. All the cytokinins tested (kinetin, zeatin, iP, and BA) gave a good shoot proliferation response. The optimal concentrations for shoot proliferation of each of the cytokinins tested were: zeatin (0.5 mgl^{-1}), kinetin (1.5 mgl^{-1}), iP (1.0 mgl^{-1}) and BA (1.5 mgl^{-1}). In combination with auxins, the optimal combinations were kinetin/NAA (2.0/0.1 mgl^{-1}), kinetin/IBA (1.5/1.0 mgl^{-1}), zeatin/IBA (1.0/0.5 mgl^{-1}), zeatin/NAA (1.0/1.0 mgl^{-1}), BA/IBA (1.0/1.0 mgl^{-1}), BA/NAA (1.5/0.1 mgl^{-1}). Although it gave the highest number of shoots per explant, BA was responsible for hyperhydricity.

Temperature and sucrose also influenced shoot proliferation. The optimal temperature was 25°C, while 30 gl^{-1} was the optimal concentration of sucrose for shoot proliferation. Plants rooted well in plant growth regulator-free MS medium. Amongst the potting mixtures tested, soil: sand: vermiculite (1:1:1 v/v) was the best with 98 % plantlet survival.

In the second part of this project, *Platycerium bifurcatum* cultures were established using leaf explants. The basal medium was MS medium (MURASHIGE and SKOOG, 1962), supplemented with 100 mgl^{-1} myo-inositol and 30 gl^{-1} sucrose. For bud initiation, 1.0 mgl^{-1} BA was used, while 0.8 % agar was used as the gelling agent. Three different strengths of MS medium (full, half, and one-quarter strength) without plant growth regulators were tested for further bud growth and development. Half-strength MS proved to be the best for further

bud growth and development. Rooting was best achieved in one-quarter strength MS medium without plant growth regulators. *In vitro* grown plantlets were successfully acclimatized using peat as the potting medium.

CONTENTS

2. Shoot Culture of *Aloe polyphylla*

3. Tissue culture of *Platycerium bifurcatum*

LIST OF ABBREVIATIONS USED

BA	Benzyladenine
iP	isopentenyladenine
K	Kinetin
Z	Zeatin
IBA	indole-3-butyric acid
NAA	∝ - naphthaleneacetic acid

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Chapter One

General literature review

1.1 Literature review of the genus *Aloe*

Introduction

The importance of plants to mankind cannot be over emphasised. We all depend upon plants for numerous needs like food, medicine, clothing, fuel and furniture. It should not be surprising therefore that much human endeavour has been aimed at producing and improving useful plants. Important in this endeavour has been the development of techniques for cultivating plant cells and tissues *in vitro* – plant tissue culture.

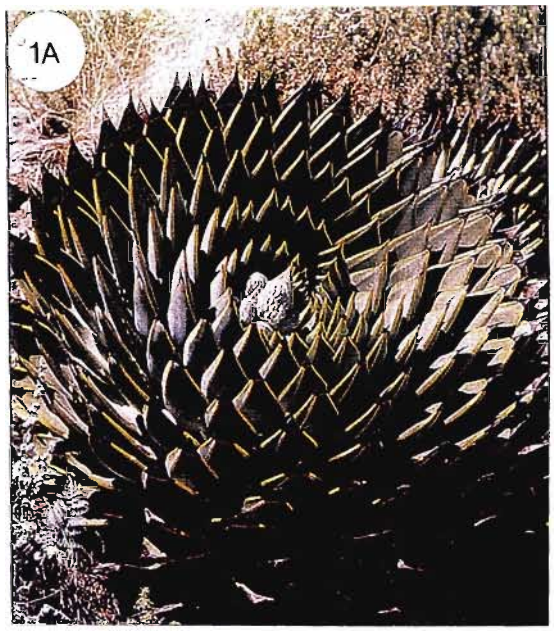
Plant tissue culture is a technique through which any plant part is cultured on a sterile nutrient medium in controlled light and temperature with the purpose of obtaining growth. The idea of plant tissue culture originated from the cell theory that was formulated by Schwann in 1839. Tissue culture techniques have for decades played a great role in the micropropagation of horticultural and ornamental plants. In fact, the first ever successful plant tissue culture was achieved in horticultural plants (ALTMAN and ZIV, 1997). These techniques have been widely used in disease elimination and vegetative propagation (HUSSEY, 1979).

Tissue culture propagation presents one solution to the problems encountered with conventional propagation. It usually ensures that the desired characteristics of a selected plant or plants are retained through clonal propagation. The rate of multiplication of plants has also been shown to be enhanced considerably using this method. It also allows year round production of plants. In commercial nurseries, it can also be used to rationalize the growing space usually constrained for the preservation of stock plants. In addition, when properly accomplished,

tissue culture may be employed for the reproduction and maintenance of disease-free plants (MURASHIGE, 1974). In view of the above benefits of tissue culture, experimentation on the *in vitro* propagation of *Aloe polyphylla* was embarked on. It was expected that in this way the devastation of native populations could be averted.

Plants belonging to the family Liliaceae are cultured for their high medicinal and ornamental value (VIJ *et al.*, 1980). *Aloe*, a member of the Asphodelaceae of the Liliaceae is cultivated in gardens for its unassuming succulent foliage as well as for aloin – an important drug (ROY and SARKAR, 1991). Although the genus *Aloe* has for many decades been recognised for its medicinal and ornamental values, it is only since research activities have disclosed that some members of this genus possess some medicinal compounds, that an interest in the pharmacological potential of these plants has developed. This has resulted in an increasing demand for aloes. In the case of *Aloe polyphylla,* and in fact for many threatened species, no method has been devised to ensure a continuous supply of these plants for commercial and research activities. Wild resources of many species are rapidly declining.

Aloe polyphylla (spiral aloe), also known as the Kharetsa, belongs to the family Asphodelaceae. This species of *Aloe* is a succulent perennial with a rounded rosette of 75-150 mostly erect leaves measuring up to 80 cm across; arranged in five spiral rows, clockwise or counterclockwise **(Fig. 1A)**. The grey-green coloured leaves are egg-shaped and very fleshy, 20-30 cm long and 6-10 cm broad when mature, nearly flat above and unevenly ridged below, and with rather soft white teeth on the margin. A flowering shoot extends 50-60 cm high, branching from near the base, with flowers crowded on the branch tips (**Fig. 1B**). Each flower has a narrow, triangular bract 2-3 cm long, and a cylindrical corolla 45-55 mm long. The flowers can be pale red to salmon pink or, very rarely,



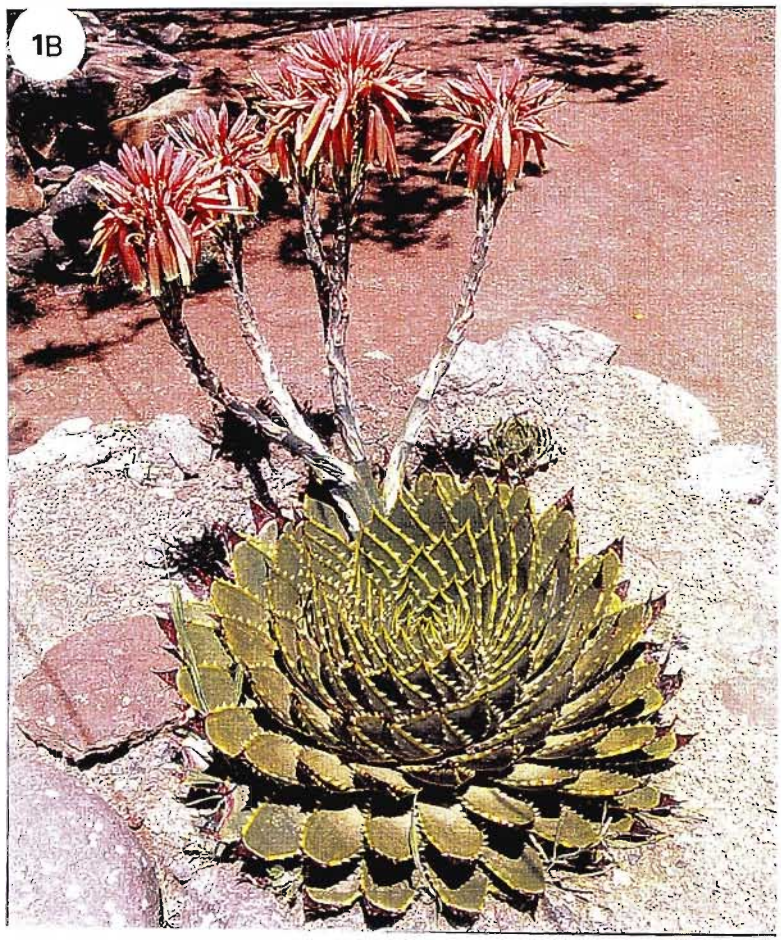


Figure 1: The morphology of *Aloe polyphylla*. **(A)** showing the clockwise arrangement of the leaves, **(B)** the counterclockwise arrangement of leaves and a flowering shoot with the arrangement of flowers at the tip of the inflorescence.

yellow. Flowering occurs from August through December, peaking in September and October (EMANOIL, 1994).

Aloe polyphylla, one of the most spectacular plants of southern Africa, is found in Lesotho, with a major concentration in the Thaba Putsoa Range and Maseru area of the Drakensberg mountains. It grows at elevations of 2230-2720 m on steep basalt slopes with loose rock. It thrives in areas where its roots are kept moist in summer by a continual flow of water, where there are mostly low shrubs, and where rainfall is around 1100 mm annually.

- Aloe Species have long been known for their medical properties leaf juice of aloe have been used medically for centuries

1.2 Horticultural and other uses of *Aloe*

Apart from their ornamental value, *Aloe* species have long been known for their medicinal properties. These plants have been cultured recently for their high medicinal and ornamental value. Leaf juice of Aloe have been used medicinally for centuries (VAN WYK and SMITH, 1996). Amongst the species known to possess some medicinal properties are *Aloe barbadensis, Aloe ferox, Aloe aristata, Aloe candelabrum, Aloe coopperi, Aloe maculata, Aloe sessiliflora, Aloe marlothii,* and *Aloe linearifolia* (VAN WYK *et al.*, 1997; HUTCHINGS *et al.*, 1996).

The medicinal substance ('Aloe') in the leaf juice of *Aloe vera* is widely used in the cosmetic and pharmaceutical industries (VIJ *et al.*, 1980). In traditional medicine, the leaves and roots of most species when boiled in water are taken as a laxative. They are also taken for Disease like arthritis, eczema, conjunctivitis, hypertension and stress. Leaf sap of several species is applied externally to treat skin irritations, bruises and burns. The leaves of *Aloe marlothii* are popular in snuff mixtures. The main purgative element is the anthrone C-glycoside aloin, while the wound-healing properties are attributed to glycoprotein and to hydrating, insulating and protective effects (HUTCHINGS *et al.*, 1996; VAN WYK *et al.*, 1997). Due to the belief by locals from Lesotho that *Aloe polyphylla* has some medicinal properties, they chop off the leaves and place them in water for poultry

which improve

consumption (KOOPOWITZ and KAYE, 1990). They also make a kind of concoction with the leaves together with other herbs which they also believe improves one`s immune system when consumed. However, these claims are yet to be proved scientifically since no study has been done to confirm these beliefs.

1.3 Conservation

With its large rosette of triangular grey-green leaves arranged in five spiral series and coral-coloured flowers, *Aloe polyphylla* is very desirable. There has been over-exploitation of *Aloe polyphylla* in its natural habitat. The trade in wild collected plants has been a contentious issue for many decades. *Aloe polyphylla* which is the national flower of Lesotho is endangered according to World Conservation Monitoring Units` Red List of Threatened Plants. This is due to the indiscriminate collection of the wild population. Apart from the collection of plants for the ornamental trade, locals, who believe that the leaves contain medicinal properties have been chopping them up. Other factors that could have contributed to the decline in numbers of *Aloe polyphylla* include urban and industrial expansion, agricultural development, afforestation and mining activities. To protect these plants, the Lesotho government prohibits export of both seeds and plants. It is illegal to remove plants from their natural habitats. This legislation was put in place in 1938 to reduce the likelihood of extinction (EMANOIL,1994).

Conservation of these ornamentals depends on the availability of propagation material to the horticulturist. This can be realised by successful rapid cultivation of these plants to meet the ever growing demand for the horticultural trade, and also guaranteeing the availability of plant material for propagation.

1.4 Conventional propagation

Conventionally, *Aloe polyphylla* is propagated both sexually and vegetatively. Sexually, it is propagated through seeds. Though it produces a large number of

seeds, only about half are viable owing to the fact that its pollinator, the Malachite sunbird, is endangered. Although sexual propagation is regarded as a most efficient and economical method of propagating plants, once seed dormancy problems have been overcome, the major disadvantage of this method is that it results in genetic variability. Besides, it is normally time and space consuming, and seasonally controlled (HARTMANN and KESTER, 1983). Vegetative propagation is through offshooting of pups which is very rare in *Aloe polyphylla*. Occasionally, a plant may split in two, and it requires two years to grow apart. Furthermore, owing to the fact that *Aloe polyphylla* apparently requires special soil and moisture for growth, conventional propagation may be difficult.

1.5 Review of tissue culture of the genus *Aloe*

Plant tissue culture is a generic description which embraces plant protoplast, plant cell, plant tissue and, plant organ culture. These various types of culture involve, as a common factor, the growth of decontaminated plant material in an aseptic environment, such as sterilized nutrient medium in a test tube or other culture vessel (DE FOSSARD, 1981). *In vitro* techniques are generally used in plant improvement which includes recovery of virus free clones, haploid cultures, embryo culture, production of chimeras, preservation of valuable germplasm, mutation initiation and selection, screening for disease, toxin and stress resistance, *in vitro* pollination and fertilization, and protoplast culture. Other uses are production of pharmaceuticals and other natural compounds; movement of plant material from one country to another, all year round production of clones, and demonstration of natural interactions between bacteria and plants such as in bacterially induced tumours and symbiotic nitrogen fixation in legumes (MURASHIGE, 1974; SPIEGEL-ROY and KOCHBA, 1977; GRESSHOFF, 1978; DE FOSSARD, 1981).

Tissue culture has been employed extensively for the rapid clonal propagation of plants (MURASHIGE, 1974; VASIL and VASIL, 1980). The technique is particularly useful for plants where the rate of multiplication is slow. Micropropagation of ornamentals is the most widely used tissue culture technique. *In vitro* cultures have been used as a tool in the acquisition of knowledge of some physiological characteristics of many plants, such as nutritional requirements, endogenous hormone production, and other developmental aspects of these plants. Not much work has been done on the *in vitro* culture of *Aloe* species owing to the fact that primary culture establishment is difficult because phenolic substances are secreted by the explants (ROY and SARKAR, 1991). The tissue culture protocols available for *Aloe* are shown in **Table 1**.

1.5.1 Shoot-tip and meristem culture

Meristem culture is a technique in which a small piece of the stem tip is cultured in appropriate nutrient medium. Only the apical dome and one or two leaf primodia are used. In shoot tip culture, a portion of the sub-apical region is involved (HUSSEY, 1979).

This technique was first developed by Morel and Martin (1952) for *Dahlia* (DEBERGH, 1994). In ornamentals, meristem culture was one of the first, and is still the most widely used procedure in commercial applications of tissue culture. The purpose of meristem culture is to free plants from virus infection. It is believed that a virus does not easily invade or reproduce in the very young cells of the shoot meristem (HUSSEY, 1979; DE FOSSARD, 1981). Besides eradicating viruses, *Pelargonium* meristem culture, combined with appropriate testing by immunofluorescence, has been effective in the production of mother plants free of *Xanthomonas pelargonii* (REUTHER, 1983). However, this technique does have some limitations. According to Debergh, *et al.* (1990) a good

Table 1 : Summary of *in vitro* work on *Aloe* species

Species	Explant	Medium & Supplements	Growth Response	Reference
Aloe arborescens **Mill.**	Axillary shoots	MS + 3% sucrose + 0.02– 0.1 mgl^{-1} kinetin + 1.8-9.2 mgl^{-1} NAA	Callus formation	Kawai *et al.* (1993)
		MS medium MS + magnetic fluid	Micropropagation 2^0 shoot production Rhizogenesis	Corneau *et al.* (1994)
Aloe pretoriensis **Pole Evans**	Leaf tissue, stem segments	Not given	Micropropagation	Groenewald *et al.* (1979)
	Seeds	LS + 0.2 mgl^{-1} 2,4-D +1 mgl^{-1} kinetin + 0.1 mgl^{-1} p-aminobenzoic acid + 100 mgl^{-1} tyrosine + 3 gl^{-1} casein hydrolysate	Callus formation that later developed into shoots and roots	Groenewald *et al.* (1976)
Aloe species (23 species)	leaves, perianth, floral stalks	MS + 1.0 mgl^{-1} NAA + 1.0 mgl^{-1} BA	Callus induction and growth	Hyashi (1987)
Aloe variegata **L.**	Stem apex	MS + supplements	Lateral bud formation	Zhao (1990)

Table 1 contd.

Species	Explant	Medium & Supplements	Growth Response	Reference
Aloe vera **L.** (*Aloe barbadensis* **Mill.**)	Apical and axillary buds	MS + 1 mgl^{-1} 2,4-D + 0.2 mgl^{-1} kinetin + 0.1 mgl^{-1} PABA + 0.02 mgl^{-1} 2,4-D + 1 mgl^{-1} kinetin	Callus induction Best for callus growth Shoot formation	Roy & Sarkar (1991)
		MS + 3 mgl^{-1} BA MS + 0.3 mgl^{-1} NAA	Bud induction Rooting	Zhou *et al.* (1999)
		MS + 3 mgl^{-1} BA + 0.2 mgl^{-1} NAA MS + 5% sucrose + 1 mgl^{-1} BA + 5 mgl^{-1} adenine + 0.2 mgl^{-1} NAA MS (half-strength) + 0.1 mgl^{-1} BA + 2-3 mgl^{-1} IBA	Adventitious shoot induction Rapid proliferation of shoots Rooting	Feng *et al.* (2000)
	Meristem tips	MS + 30% sucrose + 0.2 mgl^{-1} 2,4-D + 0.5 mgl^{-1} kinetin	Multiple shoot production	Natali *et al.* (1990)

Table 1 contd.

Species	Explants	Medium & Supplements	Growth Response	Reference
Aloe vera **L.** (*Aloe barbadensis* **Mill.**)	Multiple nodes of plants (8 - 10 cm)	Modified MS + 30% sucrose + 1.0 mgl^{-1} IBA + 1.0 mgl^{-1} IAA	. Adventitious and axillary bud growth Maximum bud growth & rooting Axillary bud formation	Meyer and Van Staden (1991)
	Stem segments	MS + 2 mgl^{-1} Zeatin + 0.5 mgl^{-1} NAA	Plantlets formed on the cell masses on callus surface	Gui *et al.* (1990)
Aloe vera **L.** & *Aloe harlona* **Danguy**	Immature inflorescence	Modified MS+ NN vitamins + 30 gl^{-1} sucrose +1.2 mgl^{-1} zeatin + 0.52 mgl^{-1} BA	Multiple shoot production Multiple shoot production	Richwine *et al.* (1995)

correlation exists between the size of the explant and the degree of contamination for a given virus. He also pointed out that the results also depend to a large extent on the physiological status of the stock plant and, thus, vary with the season. Furthermore, meristem culture has no value when it is not combined with thorough testing of the plant material afterwards (DEBERGH, 1994). Key protocols for meristem culture must be combined with some experimental treatment like chemotherapy, thermotherapy, or cryotherapy (SHARP and LARSEN, 1979; DEBERGH, 1994).

Available protocols for meristem culture of *Aloe* include the works of Natali *et al.* (1990), and Hirimburegam and Gamage (1995) with *Aloe vera* . Plantlet regeneration was achieved by both researchers on Murashige and Skoog medium (MS) (MURASHIGE and SKOOG, 1962), but with different growth regulators. Natali *et al.* (1990), used 0.25 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin while Hirimburegama and Gamage (1995), used 0.18 mg l^{-1} IAA and 2.25 mg l^{-1} BA.

1.5.2 Callus culture

Callus – proliferation of cells to form an unorganized mass of tissue - is produced when the nutrient medium contains fairly high concentrations of hormones, particularly auxins. The development of a callus from a fragment of tissue may be divided into three stages; induction of cellular division, continued proliferation and dedifferentiation, and structural and physiological re-differentiation (YEOMAN and AITCHISON, 1973). Nearly all callus cultures are derived from two major types of cells in an explant; those of vascular cambium which may already be in a state of division, and a variety of parenchyma cells which are inactive and have to be induced to divide (YEOMAN, 1973).This type of culture however, is usually avoided because of its association with genetic instability (HUSSEY, 1979; DE FOSSARD, 1981).

Polyploid cells with various types of chromosomal variations from the normal for

the species have often been reported for callus cultures, and thus plants which are induced to form from such cultures, either by induction of adventitious buds or by embryogenesis, may arise from cells of abnormal type. These abnormalities may involve flower colour, leaf shape, growth rate or other characters which would mean that the plants would be off-type. These 'disadvantages' of callus culture not withstanding, are particularly useful for analysing the influence of various factors on plant organogenesis. However, the presence of cells without the normal genome does not necessarily prevent the formation of adventitious buds and embryos with the normal chromosomal type of the species. According to Hussey (1979), changes in a callus are unpredictable and often the capacity to regenerate shoots is lost. However, the tendency for callus to develop abnormal cells varies according to the species and media on which it is grown. The fast-growing calli are generally the most likely to produce abnormal plants. On the other hand, the variation in genome could lead to the formation of interesting and variable new strains (DE FOSSARD, 1981).

According to the literature, the first organogenetically active *Aloe* callus was reported by Groenewald *et al.* (1975). Seed segments were used as explants. LS medium (LINSMAIER and SKOOG, 1965) was used with 2,4-dichlorophenoxyacetic acid (0.2 mgl^{-1}) and kinetin (1 mgl^{-1}) incorporated into the medium. After three to four weeks calli were observed at the cut edges of some of the seed fragments. After eight to ten weeks regeneration of shoots and roots were observed as well. In 1988, Castorena-Sanchez *et al.*, reported micropropagation of *Aloe vera* using callus cultures. Using DNA microdensitometry, they found that the morphogenetic ability of callus is correlated with nuclear DNA content in callus cultured *in vitro*. Micropropagation was obtained only from calli containing close to the normal 2C or 4C amount of DNA per nucleus. Cavallini *et al.* (1993) later gave credence to this work by reporting the chromosome number of six plants regenerated from *Aloe vera* callus

culture. Five of the six plants were diploid and one was tetraploid. Racchi (1988) while studying the biosynthesis of secondary products of *Aloe ferox in vitro*, achieved indirect organogenesis in callus obtained from root and embryo explants of this species. Cultures were established on NN (NITSCH and NITSCH, 1967) and MS (MURASHIGE and SKOOG, 1962) media. However, from the reported findings on callus cultures of *Aloe* species, it is evident that the plant growth regulators 2,4-dichlorophenoxyacetic acid and kinetin play major roles in callus induction and plant regeneration respectively.

1.5.3 Other *in vitro* work

Besides shoot tip, meristem and stem segments, other plant parts that have been used as explants in *in vitro* work of *Aloe* include leaf tissue, immature inflorescences, and floral stalks. Groenewald *et al.* (1979) achieved micropropagation of *Aloe pretoriensis* using leaf tissue as an explant source. Richwine *et al.* (1995) working with *Aloe vera* and *Aloe harlona* achieved multiple shoot production using immature inflorescences as explants. To achieve multiple shoot production, they used modified MS (MURASHIGE and SKOOG, 1962) medium with NN (NITSCH and NITSCH, 1967) vitamins supplemented with 1.2 mg l^{-1} zeatin or 0.52 mg l^{-1} BA.

1.6 Literature review on the genus *Platycerium*

Introduction

Platycerium bifurcatum (Cav) C. Chr. is one of fifteen species of the genus *Platycerium* belonging to the plant Polypodiaceae. It is a widespread and much diversified species occurring in southern Australia, extending beyond the tropics into the warm-temperate sub-tropical areas of eastern Australia. Generally it is found growing in areas with a pronounced dry season at an altitude of up to 2000 meters or more (HENNIPMAN and ROOS, 1982).

Platycerium bifurcatum (Cav) C. Chr. commonly known as elkhorn is an epiphytic

fern. As in other ferns, this species consists of two independent generations: the small, simple haploid gametophyte that is limited to moist environments and the large, morphological complex, diploid sporophyte. The gametophyte bears the sexual organs that carry out sexual reproduction, while the large and complex sporophyte on the other hand bears elaborate fertile leaves that facilitate spore dispersion (FERNANDEZ *et al.*, 1999). *Platycerium bifurcatum* is characterized by typical forked fertile leaves that provides excellent ornamental qualities (**Fig. 2**). The frond is dimorphic in nature, comprises the foliage fronds which become detached when they are old, and the nest fronds that remain on the plant even after they turn brown. The nest fronds help trap leaf litter from the surrounding trees (HENNIPMAN and ROOS, 1982; CHIN, 1997). According to Hoshizaki (1972) the dimorphism exhibited by the fronds is a response to the epiphytic habitat, ensuring better absorption of water and nutrients by humus collection as well as a better protection of the roots.

1.7 Horticultural and other uses of *Platycerium*

The excellent ornamental qualities provided by the leaves of this plant makes it a desirable plant. The fronds are widely used in flower bouquets and flower arrangements because of their lacy appearance. They are attached to wayside trees or used as indoor plants (CHIN,1997). Due to their attractive appearance, they have a popular following worldwide and are eagerly sought after by gardeners.

1.8 Conventional propagation

Platycerium bifurcatum is propagated conventionally both asexually (by offshoots) and sexually (by spores). Asexually, the offshoots arise as a result of branching of the short creeping rhizomes which are normally not seen as they are hidden beneath layers of nest fronds. Offshoots also arise from roots quite readily. Plants are easily increased by removal of these offshoots (HENNEN and SHEEHAN,

Figure 2: The morphology of a *Platycerium bifurcatum* plant showing its typical forked fertile leaves that provide excellent ornamental qualities.

1978; CHIN, 1997). Sexually, *Platycerium bifurcatum* is propagated through spores. This technique is most often used by horticulturists but it is generally slow (THENTZ and MONCOUSIN, 1984) and phytopathological problems are common (LANE, 1981).

1.9 Review of tissue culture of the genus *Platycerium*

In ferns, *in vitro* culture techniques have been utilised basically to study different aspects of germination, growth and development of gametophytes and sporophytes (NESTER and COOLBAUGH, 1986; HICKOK *et al.*, 1987; MILLER and WAGNER, 1987; MELAN and WHITTIER, 1990). Substantial work has also been done, using tissue culture techniques for plant regeneration and large scale generation of various fern species for the horticultural industry (HENNEN and SHEEHAN, 1978; COOKE, 1979; PADHYA and MEHTA, 1982; HIGUCHI *et al.*, 1987). The various tissue culture protocols utilized for *Platycerium* are summarized in **Table 2.**

1.9.1 Shoot - tip and meristem culture

There are a few protocols for shoot tip cultures of *Platycerium*. Amongst them is the work of Hennen and Sheehan (1978). These workers achieved plantlet regeneration from shoot tip culture of *Platycerium stemaria*. This was done on Murashige and Skoog`s (MS) medium (MURASHIGE and SKOOG, 1962) supplemented with 15 mg l^{-1} IAA, 30 g l^{-1} sucrose, and 8 g l^{-1} Difco Bacto agar. Explants produced numerous shoot primordia around the base after two months.

1.9.2 Callus culture

As far as can be established, there is only one report on cell suspension cultures obtained using gametophyte-derived callus cultures of *Platycerium coronarium* (KWA *et al.*, 1997). For the induction of callus, two-month-old aseptically grown gametophytes of *Platycerium coronarium* were cut into fine pieces and cultured

Table 2 : Summary of *in vitro* work on *Platycerium* species

Species	Explant	Media & Supplements	Growth Response	Reference
Platycerium bifurcatum **(Cav) C. Chr.**	*In vitro* grown gametophytes	Modified Miller medium (Miller and Miller, (1961) +4% sucrose	Increase in dry weight of explant (gametophyte)	Camloh and Gogala (1992)
		MS medium (half strength) +3% sucrose + 0.8% agar.	Subsequent development of sporophytes on soil surface	Knauss (1976)
	Leaves	MS + 3% sucrose + 0 or 1.1 mgl^{-1} BA	Adventitious bud development	Camloha *et al.* (1994)
		Modified MS + 0.02, 0.2, 2.0, 20 mgl^{-1} Jasmonic acid (JA)	Rhizoid and shoot development	Camloh *et al.* (1999)
		Culture medium + 0.1 mgl^{-1} Kinetin + 0.1 mgl^{-1} NAA Culture medium + active charcoal + 2 mgl^{-1} NAA	Bud induction Rooting	Jambor *et al.* (1995)
		Modified MS medium + 3% sucrose + 8 gl^{-1} agar without growth hormone	Adventitious bud formation	Camloh *et al.* (1991)
	Not given	MS + 3% sucrose + 0.9% agar + 0.1 mgl^{-1} NAA + 2.0 mgl^{-1} kinetin	Optimal shoot multiplication	Pevalek (1996)

Table 2 contd.

Species	Explant	Media & Supplements	Growth Response	Reference
Platycerium bifurcatum **(Cav.) C. Chr.**	Scales	Modified MS by Hennen and Sheehan, (1978) + 0.01 - 3 % sucrose + 0.8% agar	Outgrowth development that later developed into buds	Ambrozic and Camloh (1997)
	Sporelings	IAA or NAA in culture medium NAA in the culture medium	Multiple shoot formation Rooting	Kim *et al.* (1996)
	Spores	Knop`s medium (Miller and Greany)  Knop`s medium + Jasmonic acid (JA)	Spore germination Increase in length and number of rhizoids	Camloh *et al.* (1996)
	Spores and axillary shoots	Modified MS medium MS medium without growth regulators or MS medium (1/10 strength)	Micropropagation Rooting	Gleba and Gordzievskaya (1987)

Table 2 contd.

Species	Explant	Media & Supplements	Growth Response	Reference
Platycerium coronarium **(Koenig) Desv.**	Pieces of gametophytes	MS + 2% sucrose + 2 gl^{-1} gelrite + 4.4 mgl^{-1} 2,4-D	Callus formation	Kwa *et al.* (1997)
		MS medium + 1.1 mgl^{-1} 2,4-D + 2% sucrose	Establishment of cell suspension with increase in fresh weight	
		MS + 2.2 mgl^{-1} kinetin	Development and proliferation of callus	
		MS medium without growth hormone	Morphogenesis into gametophytes and sporophytes	
	Rhizomes and fronds	MS medium + 2% sucrose 0.2% Gelrite	Formation of bud-like structures that later developed into sporophytes	Kwa *et al.* (1995)
		MS medium + 1.1 mgl^{-1} NAA	Formation of sporophytes	

Table 2 contd.

Species	Explant	Media & Supplements	Growth Response	Reference
Platycerium species	*In vitro* grown sporophyte plants	MS medium + 3% sucrose + 0.8% agar	Multiplication of plantlets	Cooke (1979)
Platycerium stemaria **(Beauvois) Desu.**	Shoot tip	MS medium + 30% sucrose + 0.8% agar	Adventitious bud formation at the base of the explant; on roots formed in culture; and on the fronds produced on the explant in contact with the medium	Hennen and Sheehan (1978)
Platycerium superbum **(Beauvois) Desu.**	Spores	Modified de Fossards (1976) medium	Development of prothallus, sporophytic plants and adventitious buds	Bourne (1994)

on Murashige and Skoog (MS) medium (MURASHIGE and SKOOG, 1962) supplemented with 2 % sucrose and 4.4 mg l^{-1} 2,4-dichlorophenoxyacetic acid. On plantlet differentiation, cells subcultured on MS medium supplemented with 2.2 mg l^{-1} kinetin gave rise to two types of callus– dark green and pale green. The pale green callus developed into sporophytes when subcultured onto basal MS medium.

Chapter Two

Shoot Culture of *Aloe polyphylla*

2.1 Introduction

Shoot culture uses apices of lateral or main shoots, from actively growing shoots or dormant buds, as explants for shoot multiplication. This is the most important method of micropropagation. It is widely used commercially, mainly because of its independence from climate, weather and seasons (GEORGE, 1993). Shoot cultures have also been widely used for clonal propagation of ornamental flowering plants, for conservation of genetically defined stocks, and as experimental material in biochemical, physiological, and genetic investigations (BINDING and KRUMBIEGE-SCHROEREN, 1984). They are desirable due to their high multiplication rates and sufficient genetic stability in contrast to callus cultures.

2.1.1 Objectives

Aloe polyphylla, which is considered to be highly endangered (EMANOIL,1994) has once been propagated *in vitro* (ABRIE and VAN STADEN, 2001). The following study was therefore initiated in an attempt to optimize the tissue culture protocol for differentiation, regeneration, and multiplication of *Aloe polyphylla* plants. The aim of this research work however, was to optimise the different stages of a tissue culture protocol of *Aloe polyphylla* laying emphasis on multiplication and acclimatization. Considering the endangered status of this plant, a very successful optimised protocol will definitely play a major role in saving this important plant for mankind while at the same time optimizing production for horticultural exploitation.

2.2 Materials and methods

2.2.1 Decontamination procedures and aseptic techniques.

Contaminants can cause large losses during micropropagation and their control is usually the most frequent and a difficult problem encountered in micropropagation. It is very important to detect and eliminate contaminating organisms before they are transferred to many culture vessels during routine subcultures. This is of utmost importance if contamination is to be avoided.

Plants are invariably infested externally with fungi, bacteria, yeasts and animal pests (GEORGE, 1993). Other sources of contamination include the working area, culture media, and working instruments. Generally, the plant material is decontaminated with different concentrations of sodium hypochloride (NaOCl) depending on the plant material.

Prior to use, the surface of the laminar flow bench was swabbed down with 95% ethyl alcohol and the interior sprayed with the same alcohol. All glassware, instruments and media were steam-sterilized in an autoclave at a pressure of one bar and temperature of 121°C for 20 minutes. Instruments in use on the bench were placed in a beaker containing 95% ethanol and were flamed repeatedly using a spirit burner during the course of the work. A bead sterilizer was also used for the instruments. The worker`s hands and forearms were washed thoroughly with soap and water and repeatedly sprayed with alcohol during the period of work. The mouth of all culture vessels was flamed before and after positioning of the explant on the medium.

The explants used for this study were *Aloe polyphylla* shoots (2-3 cm long) taken from *in vitro* grown plantlets. Due to large contamination rates experienced in the preliminary experiments, even when other aseptic procedures were followed, explants were later re-decontaminated with 1% NaOCl (Jik at 3.5%). This was

done by dipping the explants in 1% NaOCl for three minutes with continuous agitation of the solution to ensure efficient distribution of the sterilant over the plant material. This was followed by three rinses in autoclaved distilled water. On completion of surface decontamination, the plant material, was placed on a sterile petri dish for the removal of the outer damaged material. All further dissection took place on sterile petri dishes and the explants, thus prepared, were transferred to the culture vessels containing the nutrient medium.

2.2.2 Explant source

Totipotentiality is probably characteristic of all plant cells, but its expression may be greater for particular cells. Familiarity with a cultivar`s peculiarities are frequently helpful in seeking explant sources (MURASHIGE, 1976). The choice of a suitable explant is essential for successful tissue culture. The size, origin, and physiological status of an explant can also affect its response in culture. For this research project, shoots, 2-3 cm long with a maximum of five leaves taken from *in vitro* grown plantlets were used.

2.2.3 Media and supplements

For plant tissue culture, many different media have been developed. The origin of these media was mostly determined by the different nutrient requirements of different plants. The choice of a medium for a plant normally depends on media used for closely related species. Generally, these media are composed of a mixture of inorganic nutrients and organic components which include sucrose and sometimes plant growth regulators, depending on what kind of response the researcher wants to achieve. Apart from the common constituents of a medium, some unidentified supplements such as yeast extract, juices, pulps and extracts from various fruits have been used to improve growth and development. The pH of the medium is usually adjusted by the addition of dilute hydrochloric acid (HCl) or sodium hydroxide (NaOH) to a pH range of between 5 and 6 prior to

autoclaving.

The standard culture medium used throughout this study contained full strength of the macro-nutrients, micro-nutrients and vitamins as described by Murashige and Skoog (1962). Details of these constituents are presented in **Table 3.** For the present research work, all constituents of this medium besides sucrose were made up as stock solutions. These solutions were obtained by dissolving the required amounts of analytical grade macro-nutrients, micro-nutrients and vitamins in distilled water, and making the final volume up to 1000 ml (1 litre). All stocks were stored in glass containers at 5°C. Those stocks that contained light-sensitive constituents such as vitamin complexes, were stored in containers wrapped in aluminium foil to exclude light.

To obtain the complete culture medium, stock solutions were combined in volumes as shown in the last column of **Table 3.** This was supplemented with 30 gl^{-1} sucrose, 0.1 gl^{-1} myo-inositol, and made to volume with distilled water. The pH of the medium was adjusted to 5.8 using sodium hydroxide (NaOH). To each litre of medium, 0.8 g of agar was added to solidify it. This was dissolved in the medium by steaming in a microwave for about ten minutes prior to dispensing into the culture vessels. All cultures were initiated in 25 mm by 80 mm glass tubes, each glass tube containing 12 ml of medium. Re-decontaminated shoots were inoculated onto the basal medium and tubes sealed with Cap-O-Test tops. Cultures were incubated at 25 ± 2°C with continuous flourescent light at a photon flux density (400-700 nM) of 30 - 50 µmol m^{-2} s^{-1}.

Shoot explants were placed on the basal medium, each supplemented with various concentrations of plant growth regulators - benzyladenine (BA), kinetin, zeatin or isopentenyladenine (iP). In another set of experiments explants were again placed on the basal medium supplemented with various concentrations of plant growth regulators in the following combinations: kinetin and ∝-

Table 3: Revised MURASHIGE and SKOOG (1962) nutrient medium

STOCK SOLUTION	CHEMICAL	MASS g 500 ml^{-1} STOCK SOLUTION	ml STOCK SOLUTION USED l^{-1} MEDIUM
1	NH_4NO_3	82.5	10
2	KNO_3	47.5	20
3	$CaCl_2$	17.2	10
4	$MgSO_4.7H_2O$	18.5	10
5	NaFeEDTA	2.0	10
6	KH_2PO_4	8.5	10
7	H_3BO_3 $MnSO_4.4H_2O$ $ZnSO_4.7H_2O$ KI	0.310 1.115 0.430 0.04	10
8	$NaMoO_4.2H_2O$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$	0.0125 0.0013 0.0013	10
9	Thiamine. HCl Nicotinic acid Pyridoxin. HCl Glycine	0.005 0.025 0.025 0.100	10
Additional	Sucrose Agar Myo - inositol		30 gl^{-1} medium 8 gl^{-1} medium 0.1 gl^{-1} medium

pH adjusted to 5.8 with NaOH

naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA), zeatin and NAA or IBA, BA and NAA or IBA. Cultures were incubated at 25 ± 2°C with continuous flourescent light at a photon flux density (400 - 700 nM) of 30 - 50 µmol m^{-2} s^{-1}. Eight replicates were used per experiment.

In yet another set of experiments, to determine the effect of sucrose concentration on shoot proliferation, explants were placed on the basal medium (full strength MS medium) supplemented with 0.5 mgl^{-1} of zeatin and different concentrations of sucrose. Cultures were incubated at 25±2°C under continuous flourescent light at a photon flux density (400 - 700 nM) of 30 - 50 µmol m^{-2} s^{-1}. Eight replicates were used per treatment.

2.2.4 Environmental conditions

The major factors that are always considered in the culture environment are temperature, light, and relative humidity. The average constant growth room temperature is normally 25°C, (GEORGE, 1993). However, explant establishment, culture growth, plantlet development and morphogenesis have all been found to be temperature-dependent. In the past, relative humidity was never thought to be an important environmental factor, until it was discovered to have an effect on the hyperhydricity of cultured shoots and plantlets. Thus relative humidity within a culture vessel depends on its temperature and that of the medium (GEORGE, 1993). Light is considered as a complicated factor which can be divided into light intensity, photoperiod, and spectral composition. Normally, light is needed for photosynthesis, photomorphogenesis, and photoperiod. Since cultures are supplied with enough carbohydrate, and phototropism is not of great importance, light is critically important in plant tissue culture for photomorphogenesis.

In this part of the research work an effort was made to study the effect of

temperature on shoot proliferation in order to determine the optimal temperature for this activity. Explants were cultured on the basal medium supplemented with 0.5 mg l^{-1} zeatin. Cultures were incubated under standard light conditions at different temperatures. Eight replicates were used per treatment.

2.2.5 Acclimatization

Acclimatization or "hardening-off" is a process by which *in vitro* propagated plants are made to adapt to an *in vivo* environment. It is basically concerned with rooting, either *in vitro* or *in vivo*, and transfer to non-sterile conditions with humidity control and temperature (DUNSTAN and TURNER, 1984). This is very important considering that the waxy cuticle and stomata of *in vitro* grown plants are inadequate or non-functional (SUTTER and LANGHANS, 1979; BRAINERD and FUCHIGAMI, 1981; WETZSTEIN and SOMMER, 1982). However, the rate of transpiration is always high in such leaves when subjected to the variable humidity of the *in vivo* environment. There is also reason to believe that the high sucrose and salt medium that is often used with *in vitro* grown cultures limits the photoautotrophic capacity of leafy shoots (WETZSTEIN and SOMMER, 1982).

In vitro rooted plantlets of *Aloe polyphylla* were first washed in water to remove excess agar since it has been found that sucrose and other organic compounds trapped by agar in the proximity of roots cause plantlets to be infected by disease causing organisms or be damaged by toxic microbial metabolites (GEORGE, 1993). Plantlets were then potted in different planting mixtures – full potting soil; 1 potting soil: 1 sand: 1 vermiculite (v: v: v); 1 sand: 1 peat: 1 perlite (v: v: v). Plants were kept in the mist house (with over-head sprinklers) and at a temperature of 24°C ± 2°C for five weeks after which they were transferred to the green house.

2.3 Results and Discussion

2.3.1 Decontamination procedures

As the explants were taken from *in vitro* grown plantlets, it was deemed unnecessary to decontaminate them. However, when the first set of shoot cultures was established, many shoots were lost due to fungal contamination. An attempt was made to save the contaminated cultures by re-decontaminating them in 1% and 2%, NaOCl and spore-kill respectively. This led to the death of most of the explants while some of the surviving ones were still contaminated.

At this stage, it became inevitable to discard this set of explants and a new set of explants was employed. The new set of explants from the same source was now successfully decontaminated by dipping them in 1% NaOCl for five minutes followed by three rinses in autoclaved distilled water. They were successfully established without observing any contamination. In subsequent experiments, this procedure was adopted and it provided good results.

2.3.2 Media and supplements

Since this study was concerned with optimizing the tissue culture protocol for *Aloe polyphylla,* the number of healthy shoots produced by individual plant growth regulators or in combination in the basal medium was recorded.

2.3.2.1 Effects of plant growth regulators on regeneration

The response of explants to different plant growth regulators singly or in combination varied. The response with respect to shoot proliferation (both axillary and adventitious shoots) is shown in Figures 3 to 14 and Plates 1 to 10. Shoots were produced on explants with the inclusion of the cytokinins BA, kinetin, iP, or zeatin singly or in combination with the auxins IBA or NAA in the basal medium. Both axillary and adventitious shoots were formed.

However, of all the cytokinins tested, BA gave the highest shoot proliferation response. After about 15 days the base of the explants became swollen forming adventitious buds which later developed into shoots. A yellowish callus was observed at the base of the explants from where most of the adventitious shoots seemed to originate. Callus formation increased with an increase in BA concentration. BA (1.5 mg l^{-1}) in the basal medium gave the highest average number of shoots (12 shoots per explant) (**Fig.3**). This was not statistically different from the 10 and 9 shoots per explant produced by 0.5 mg l^{-1} and 1.0 mg l^{-1} BA respectively in the basal medium. Hyperhydricity was very pronounced especially in cultures on media with 1.0, 1.5, 2.0, or 3.0 mg l^{-1} BA. Most of the shoots were stunted and light green, quite unlike cultures with 0.5 or 0.1 mg l^{-1} BA (**Plate 1**). Generally, cultures on media with 0.5, or 0.1 mg l^{-1} looked healthier than cultures with higher concentrations of BA. Browning occurred in about 7% of cultures.

With kinetin alone in the culture, multiple shoot production was observed (**Plate 2**). A yellowish callus was also noticed at the base of the explant which tended to increase in mass with an increase in kinetin concentration. This was first observed in cultures with 1.5 mg l^{-1} kinetin which happens to be the optimal concentration for shoot proliferation (8 shoots per explant) (**Fig.4**). However, callus development was much more prominent in the culture with higher concentrations of kinetin (2 and 3 mg l^{-1}) (**Plate 3.**). Both axillary and adventitious shoots were formed, the adventitious shoots originated from the base of the explants. Of all the shoots produced in cultures with 1.5, 2.0 and 3.0 mg l^{-1} of kinetin, 6, 12, 25 % were adventitious in origin respectively. Browning was witnessed in very few cultures contrary to its severity in *Aloe vera* cultures reported by Natali *et al.* (1990), while hyperhydricity developed sporadically in cultures with 2 and 3 mgl^{-1} kinetin.

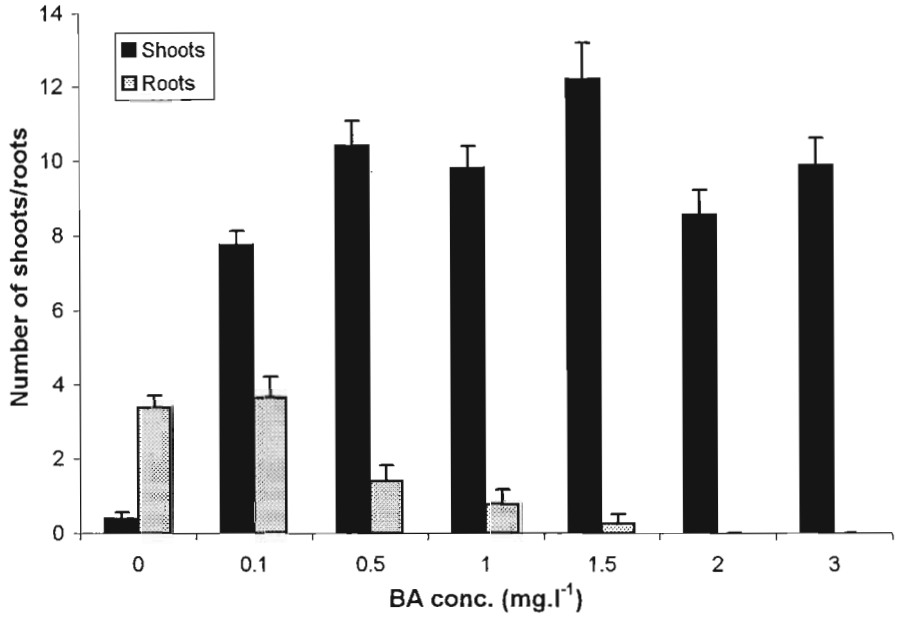


Figure 3 : Effects of various concentrations of BA on shoot and root proliferation of *Aloe polyphylla in vitro*

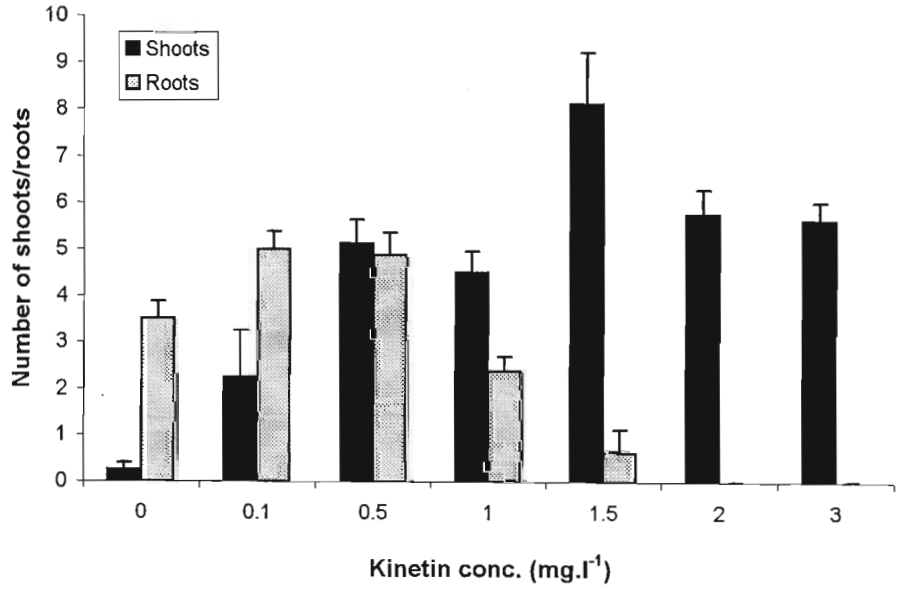


Figure 4: Effects of various concentrations of kinetin on shoot and root proliferation of *Aloe polyphylla in vitro*

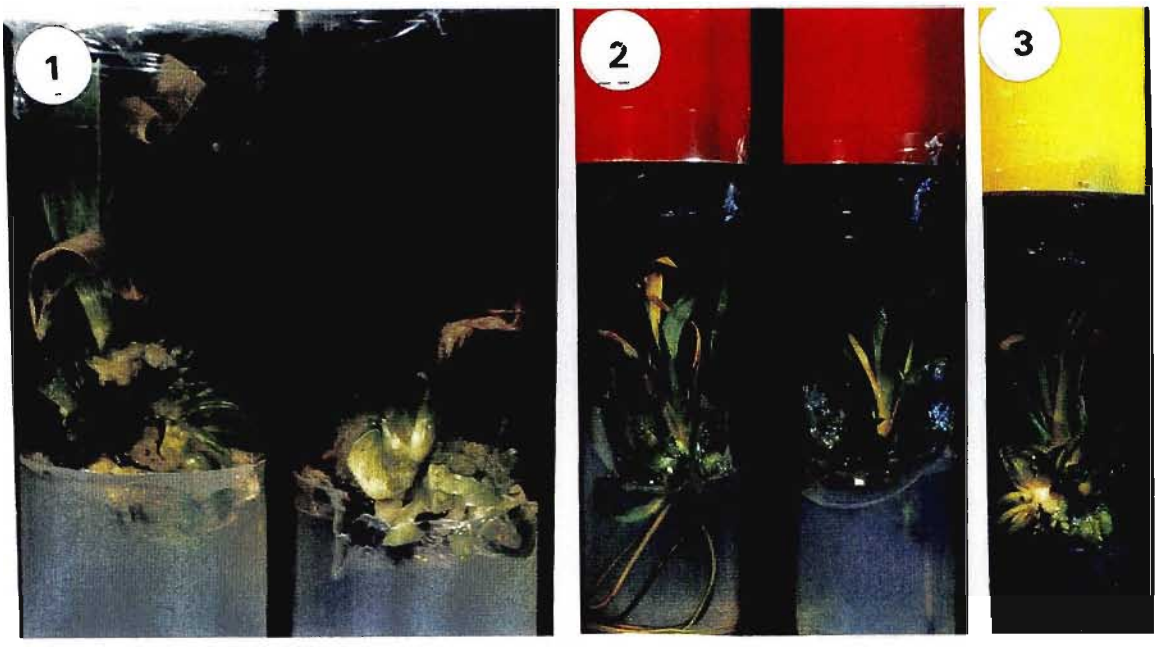


Plate 1: Multiple stunted and light green shoots obtained after about 5 weeks of treating cultures with 3.0 mgl^{-1} of BA.

Plate 2: Multiple shoot production with 1.5 mgl^{-1} kinetin in the basal medium.

Plate 3: Callus production at the base of explant with 3.0 mgl^{-1} kinetin in the basal medium.

Unlike other cytokinins the optimal concentration for shoot proliferation with zeatin alone in the basal medium was 0.5 mg l^{-1} producing an average of 7 shoots per explant (**Fig. 5**); statistically this was different from other concentrations. Shoots were healthy and dark green (**Plate 4A**). Again formation of a yellowish callus was observed with 0.5 mg l^{-1} zeatin. Callus formation increased with an increase in zeatin concentration. The number of adventitious shoots observed also increased with an increase in cytokinin concentration. Cultures containing 0.5 mg l^{-1} zeatin had the least percentage of adventitious shoots (5 %) of the total number of shoots formed, compared with the 88 % occurring in cultures with 3.0 mg l^{-1} of zeatin. Hyperhydricity was observed in about 2 % of cultures, occurring in cultures containing higher concentrations of 3 mg l^{-1} of zeatin.

With the cytokinin iP alone in the basal medium, similar results to BA were obtained except that fewer shoots were formed and hyperhydricity was not as pronounced as in cultures with BA. The optimal iP concentration for shoot proliferation was 1.0 mg l^{-1} producing an average of 8 shoots per explant (**Fig. 6**), **(Plate 4B)**.

Multiple shoot formation was observed in cultures with the cytokinins - BA, zeatin, or kinetin in combination with the auxins - NAA or IBA. Shoot proliferation activity of kinetin alone in the basal medium was better than when it was used in combination with either IBA or NAA. The optimal combination of both (ie kinetin/NAA and kinetin/IBA) for shoot proliferation was 2.0/0.1 and 1.5/1.0 mg l^{-1} respectively, producing an average number of 5 and 7 shoots per explant respectively (**Figs. 7 & 8), (Plate 5A & 5B).** There was a sporadic occurrence of hyperhydricity and browning. No development of the yellowish callus was noticed. Zeatin/IBA and zeatin/NAA combinations, like other cytokinin/auxin combinations tested, also produced a good number of healthy shoots. The optimal zeatin/IBA and zeatin/NAA combinations were 1.0/0.5 mg l^{-1} (7 shoots per explant) and 1.0/1.0 mg l^{-1} (7 shoots per explant) (**Figs. 9 & 10).** Browning and hyperhydricity

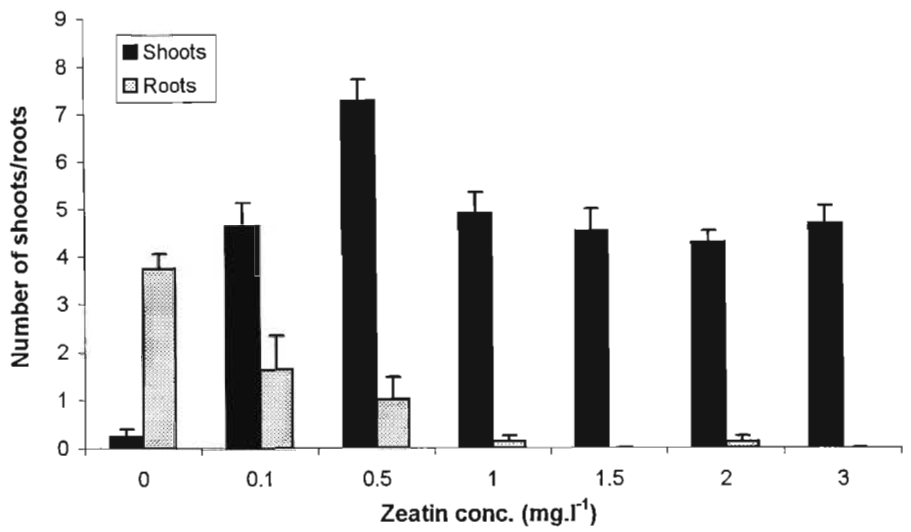


Figure 5: Effects of various concentrations of zeatin on shoot and root proliferation of *Aloe polyphylla in vitro*

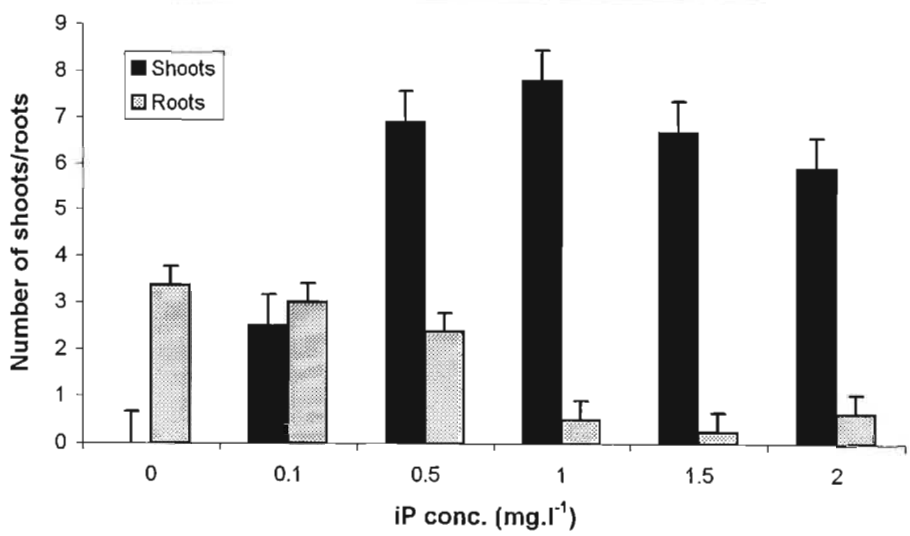
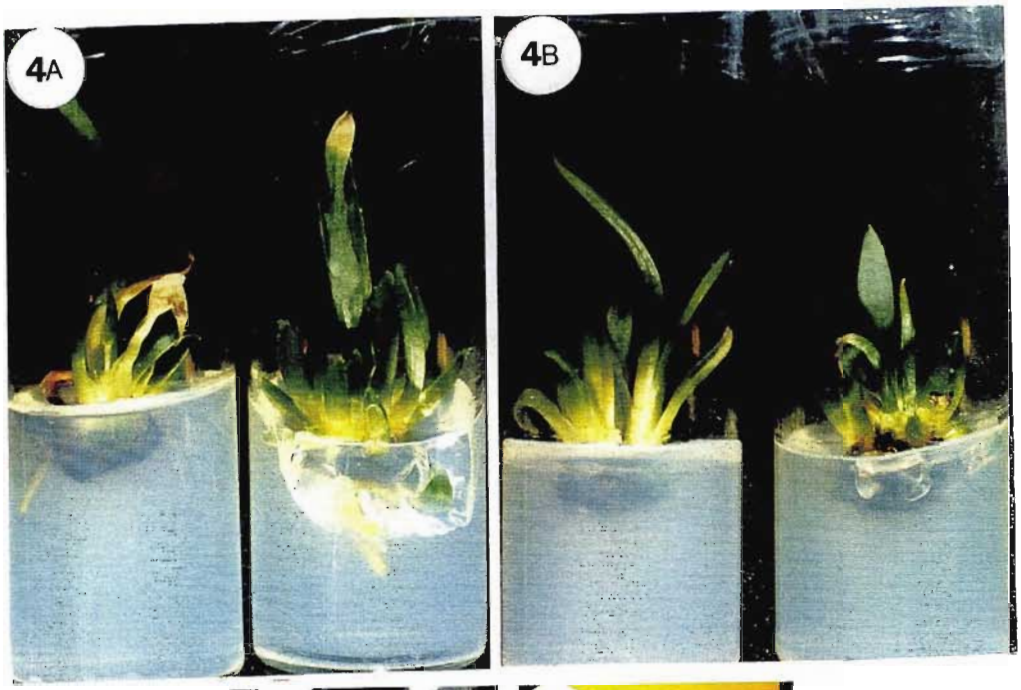


Figure 6: Effects of various concentrations of iP on shoot and root proliferation of *Aloe polyphylla in vitro*



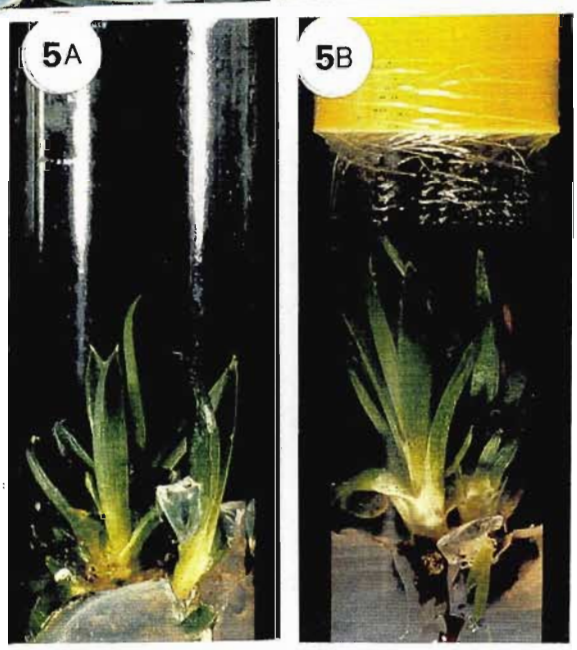


Plate 4: Multiple shoot production with cytokinins tested. (**A**) 0.5 mgl^{-1} of zeatin, (**B**) 1.0 mgl^{-1} of iP.

Plate 5: Multiple shoot production obtained with different combinations of kinetin and auxin in the basal medium. (**A**) kinetin/NAA (2.0/0.1 mgl^{-1}), (**B**) kinetin/IBA (1.5/1.0 mgl^{-1}).

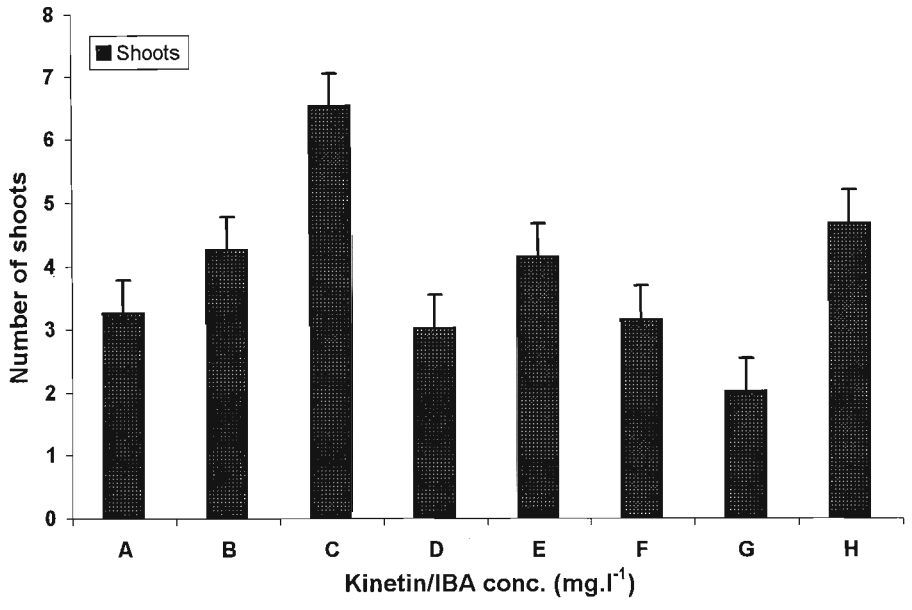


Figure 7: Effect of various combinations of kinetin/IBA on shoot proliferation of *Aloe polyphylla in vitro*

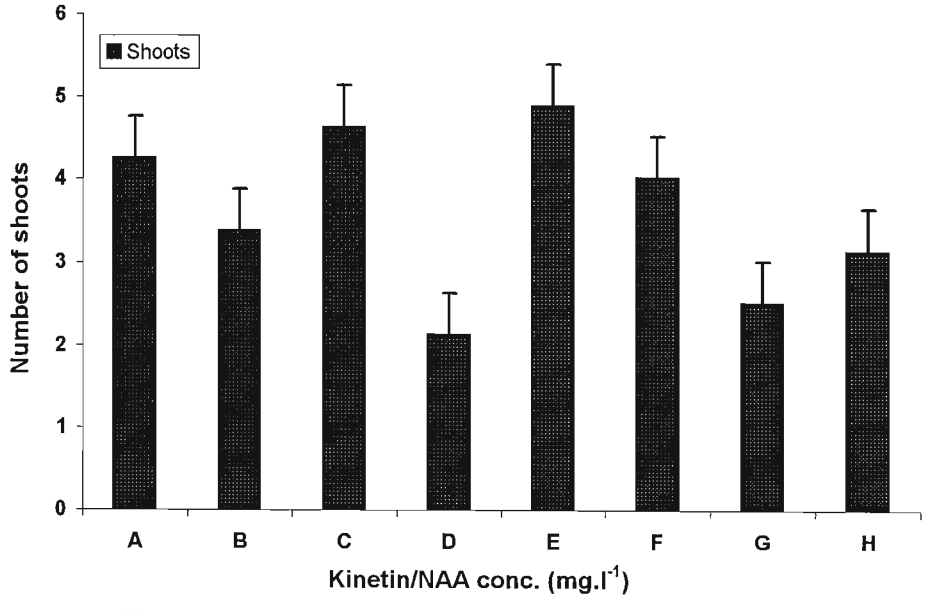


Figure 8: Effect of various combinations of kinetin/NAA on shoot proliferation of *Aloe polyphylla in vitro*

Kinetin/NAA & Kinetin/IBA (mg/l)

A - 1.5/0.1	**C - 1.5/1.0**	**E - 2.0/0.1**	**G - 2.0/1.0**
B - 1.5/0.5	**D - 1.5/2.0**	**F - 2.0/0.5**	**H - 2.0/2.0**

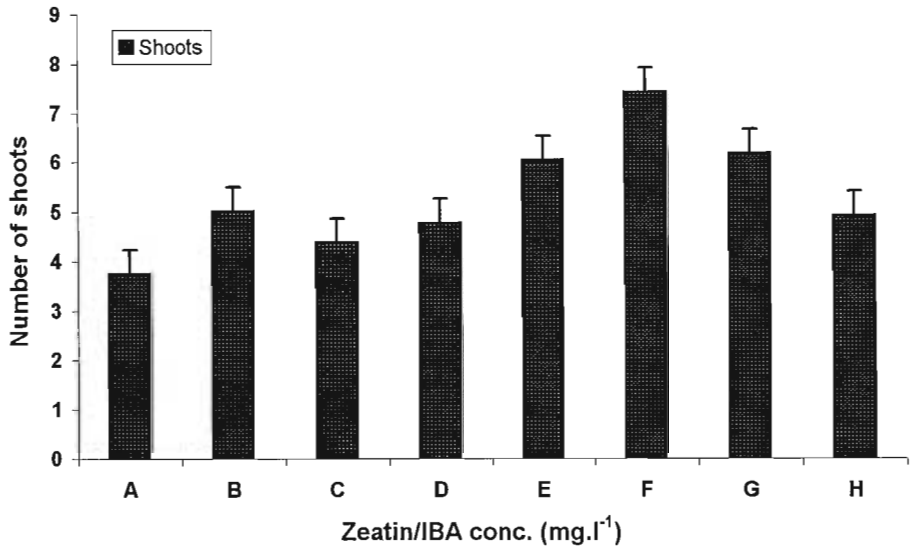


Figure 9: Effect of various combinations of zeatin/IBA on shoot proliferation of *Aloe polyphylla in vitro*

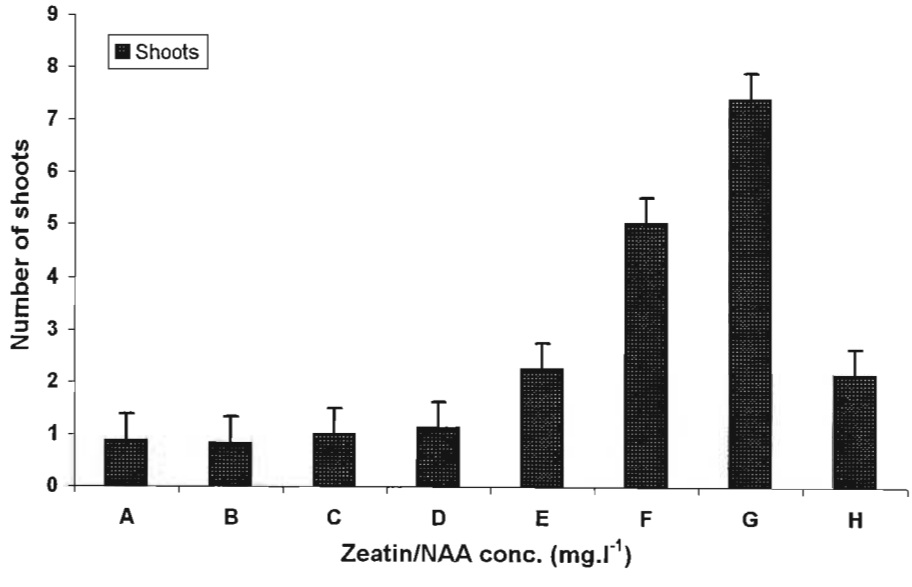


Figure 10: Effect of various combinations of zeatin/NAA on shoot proliferation of *Aloe polyphylla in vitro*

Zeatin/NAA & Zeatin/ BA (mg/l)

A - 0.5/0.1	**C - 0.5/1.0**	**E - 1.0/0.1**	**G - 1.0/1.0**
B - 0.5/0.5	**D - 0.5/2.0**	**F - 1.0/0.5**	**H - 1.0/2.0**

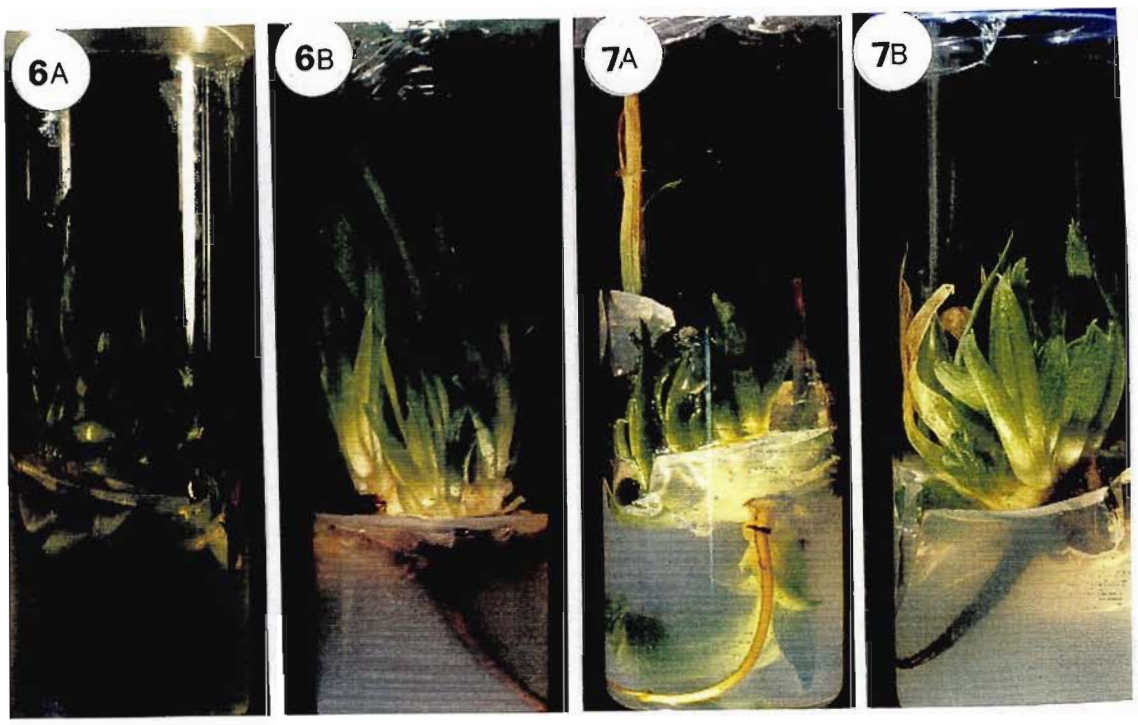


Plate 6: Multiple shoot production obtained with different combinations of zeatin and auxin in the basal medium. (**A**) zeatin/NAA (1.0/1.0 mgl^{-1}). (**B**) zeatin/IBA (1.0/0.5 mgl^{-1}).

Plate 7: Multiple shoot production obtained with different combinations of BA and auxin in the basal medium. (**A**) BA/NAA (1.5/0.1 mgl^{-1}), (**B**) BA/IBA (1.0/1.0 mgl^{-1}).

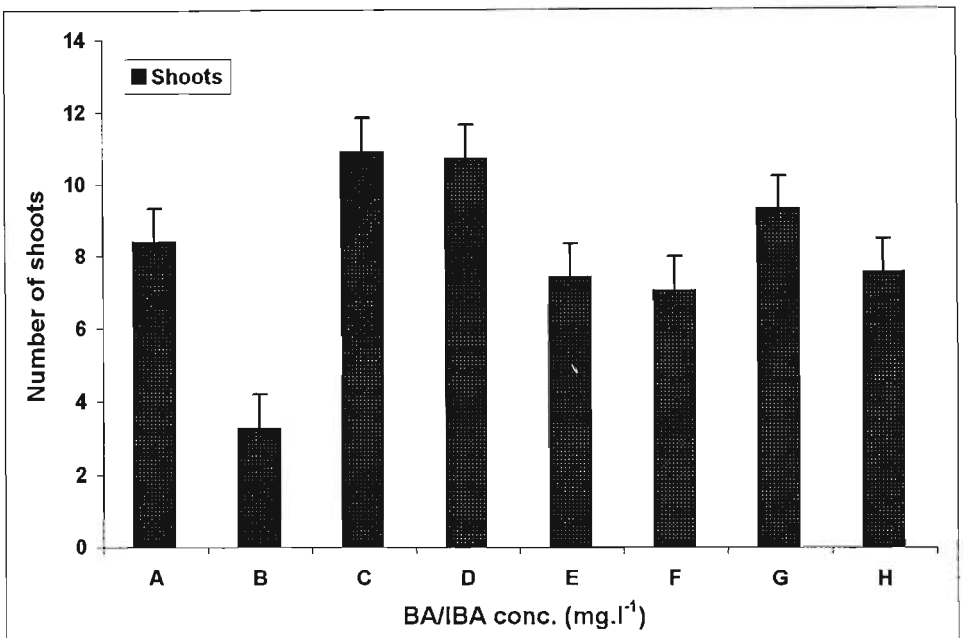


Figure 11: Effect of various combinations of BA/IBA on shoot proliferation of *Aloe polyphylla in vitro*

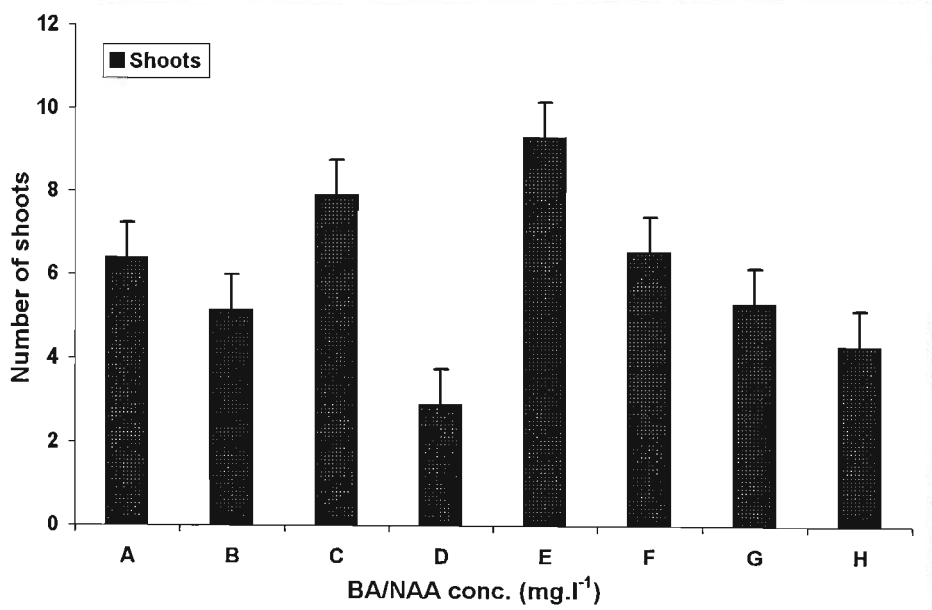


Figure 12: Effect of various combinations of BA/NAA on shoot proliferation of *Aloe polyphylla in vitro*

BA/NAA & BA/IBA (mg/l)

A - 1.0/0.1	C - 1.0/1.0	E - 1.5/0.1	G - 1.5/1.0
B - 1.0/0.5	D - 1.0/2.0	F - 1.5/0.5	H - 1.5/2.0

were not observed. Shoots produced were healthy, having a dark green appearance (**Plate 6A & 6B).** Multiple shoot formation was also observed with BA/IBA and BA/NAA combinations. The optimal combinations were 1.0/1.0 mg l^{-1} (11 shoots per explant) and 1.5/0.1 mg l^{-1} (9 shoots per explant) (**Figs. 11 & 12), (Plate 7A & 7B)**.

Few culture media have been used in tissue culture of species of *Aloe*, with different explants employed. Shoot and meristem tip explants of *Aloe* have been cultured on MS (MURASHIGE and SKOOG, 1962) medium (NATALI *et al.*, 1990; ROY and SARKAR,1991; GUI *et al.*, 1990; ZHAO, 1990; KAWAI *et al.*, 1993; CORNEANU *et al.*, 1994; HIRIMBUREGAMA and GAMAGE, 1995) and modified MS medium (MEYER and VAN STADEN, 1991; RICHWINE *et al.*, 1995). The number of proliferating shoots is an important factor to consider when developing an optimal tissue culture protocol. In this research, the presence or absence of a certain plant growth regulator influenced the number of proliferating shoots. The results of this study showed that shoot proliferation was improved by the addition of plant growth regulators, although some explants formed shoots on hormone-free medium. The potential of these explants to produce shoots in hormone- free medium could probably be attributed to the presence of endogenous hormones.

Results showed that the presence of cytokinins – BA, kinetin, zeatin or iP alone or in combination with auxins – NAA or IBA in the basal medium increased shoot proliferation activity at different levels. Previous workers reported that cytokinins alone or in combination with auxins positively influenced shoot proliferation in *Aloe* species (GUI *et al.*, 1990; NATALI *et al.*, 1990; ROY and SARKAR, 1991; HIRIMBUREGAM and GAMAGE, 1995; RICHWINE *et al.*, 1995; FENG *et al.*, 2000). Auxins (NAA and IBA) alone in the basal medium did not promote shoot proliferation unlike the report of MEYER and VAN STADEN (1991).

Results also showed that a high level of cytokinin (2 and 3 mg l^{-1}) adversely affected shoot proliferation, usually promoting the formation of a yellowish callus at the base of explants. BA which showed the best shoot proliferation potential was associated with hyperhydricity, and most of the shoots so formed were stunted. Hyperhydricity was observed in cultures with 1.0, 1.5, 2.0, or 3.0 mg l^{-1} of BA. BA is known for its hyperhydricity-inducing tendencies in shoot cultures (LESHEM *et al.*, 1988; LI *et al.*, 1997; TSAY and DREW, 1998; THOMAS *et al.*, 2000). Zeatin was another cytokinin that showed high shoot proliferation activity. Unlike BA, the shoots were elongated and hyperhydricity was very low, occurring only in cultures with 2 and 3 mg l^{-1} zeatin. The optimal concentration (0.5 mg l^{-1}) for shoot proliferation was much lower than other cytokinins. Kinetin and iP also produced good results except that iP was also associated with hyperhydricity. However, the effect was not as pronounced as for BA. The shoots were not stunted as with BA. The results also showed BA to be slightly more active with respect to number of proliferating shoots than other cytokinins tested (MURASHIGE, 1974).

Combinations of BA and NAA or BA and IBA proved to be better than BA alone in the basal medium in terms of reducing the incidence of hyperhydricity and callus formation. This could be attributed to the balancing of the cytokinin/auxin ratio. Zeatin in combination with IBA or NAA was slightly better than zeatin alone with respect to shoot formation. In contrast, kinetin alone in the basal medium was better than when in combination with either NAA or IBA with regards to shoot proliferation. Generally, there was not much difference in shoot proliferation activities between the tested cytokinins alone or when used in combination with auxins. Most previous workers reported that combinations of cytokinins and auxins were better than cytokinins alone in the basal medium with respect to shoot proliferation (NATALI *et al.*, 1990; ROY and SARKAR, 1991; HIRIMBUREGAM and GAMAGE, 1995; FENG, 2000). This contrasts with the

results of this study. It was only Richwine *et al.* (1995) who reported multiple shoot production for *Aloe vera* and *Aloe harlona* with kinetin or BA alone in the basal medium. In this study all the cytokinins singly or in combination with IBA or NAA showed multiple shoot production.

Browning was not a severe problem since the explants used were young shoots. This might be as a result of the fact that juvenile tissues tend to have a greater capacity for restoration (MURASHIGE and SKOOG, 1962) or produce less phenols (ROY and SARKAR, 1991).

2.3.2.2 Effects of sucrose on regeneration

The presence of sucrose in the basal medium had a tremendous effect on shoot proliferation. At 0% level, there was hardly any shoot proliferation (**Fig.13**), (**Plate 8A).** After about 30 days of incubation, explants showed no sign of shoot proliferation nor rooting despite the presence of 0.5 mg l^{-1} zeatin in the basal medium. With 1% of sucrose in the basal medium, after about 30 days of incubation, shoot proliferation was observed. An average of 5 shoots per explant was obtained (**Fig.13**). When the sucrose concentration was increased to 3 %, there was a significant increase in the number of shoots produced (8 shoots per explant), **(Fig.13), (Plate 8B)**, but there was no significant increase in the number of shoots obtained when the sucrose concentration was increased further to 4 % (**Fig.13**). However, when the concentration was increased to 5 % and finally 6 %, shoot proliferation declined significantly (**Fig.13)**.

Carbohydrates are generally added to culture media to serve as a carbon and energy source. Besides, they also play an important role in the regulation of the external osmotic potential. In most cultured plant tissue, sucrose is the primary substrate for respiration that produces carbon dioxide on which metabolism and growth of the tissue depends (THORPE and MEIER,1972; DODDS and

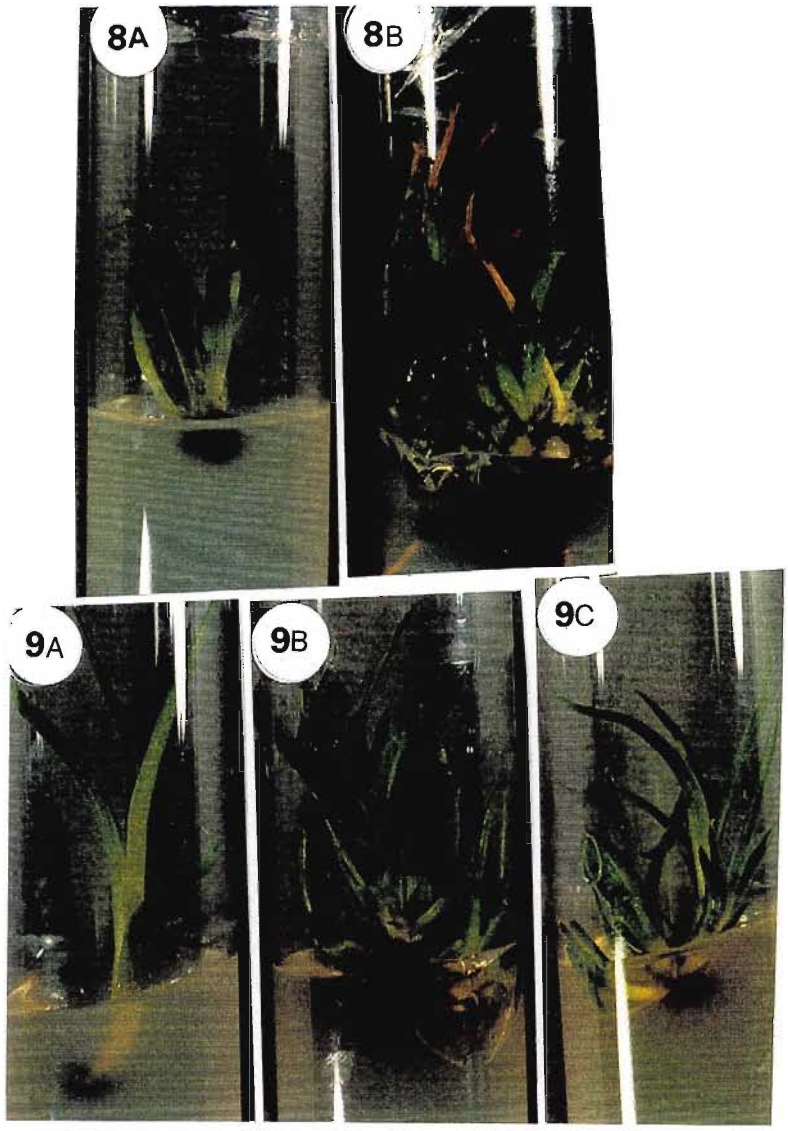


Plate 8: Effect of sucrose on shoot proliferation of *Aloe polyphylla in vitro*. (**A**) at 0 % level, no shoot proliferation, (**B**) at 3 % level, multiple shoot proliferation.

Plate 9: Effect of temperature on shoot proliferation of *Aloe polyphylla in vitro.* (**A**) at 10 ^{O}C, shoot proliferation was near zero, (**B**) at 20°C, a sharp increase in shoot proliferation, (**C**) at 30°C, shoot proliferation was inhibited.

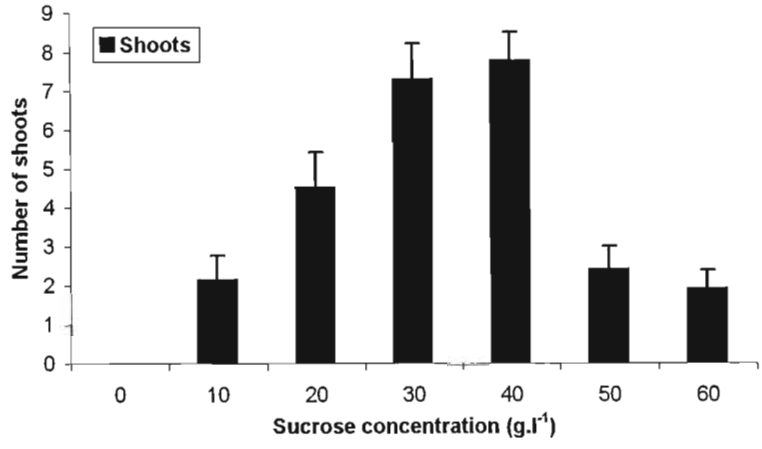


Figure 13: Effect of various levels of sucrose on shoot proliferation of *Aloe polyphylla in vitro*

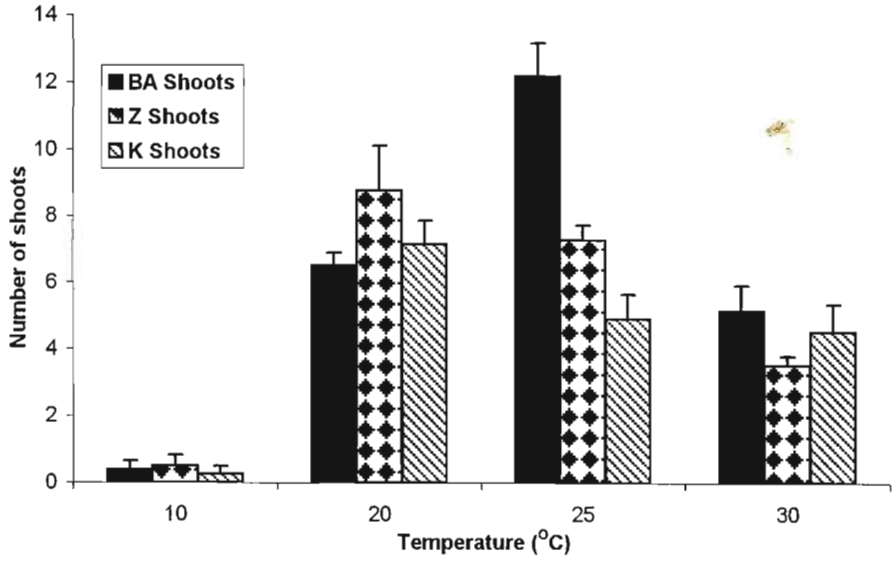


Figure 14: Effect of various temperatures on shoot proliferation of *Aloe polyphylla in vitro*

ROBERTS,1985; LANGFORD and WAINWRIGHT, 1988; GEORGE, 1993). The results of this study showed no proliferation of shoots by the explants at 0% sucrose after about five weeks. Explants did however, not die. These results were obtained irrespective of the presence or absence of 0.5 mg l^{-1} of zeatin in the medium. According to Thorpe and Murashige (1968 and 1970) the main function of carbohydrate (sucrose) in the medium is to serve as a readily available source of energy for the initiation of shoot primodia and their subsequent development.

Results also showed a marked increase in shoot proliferation by explants at sucrose concentration of 3 and 4 %. The number of shoots formed at these sucrose levels did not statistically differ. Regeneration of shoots at these levels of sucrose could be credited to the fact that metabolism and growth of the tissues were feasible due to the respiratory activities of the tissues made possible by the presence of a suitable substrate (sucrose) (THORPE and MURASHIGE, 1968, 1970). There was a significant decrease in shoot proliferation by explants at higher sucrose concentrations. Langford and Wainwright (1988) reported in their study that the greater the sucrose concentration in the medium, the less carbon dioxide was taken up per unit chlorophyll, perhaps indicating a greater chlorophyll efficiency at the lower sucrose concentrations.

2.3.3 Temperature

2.3.3.1 Effects of temperature on shoot regeneration

Temperature was observed to exert some effect on shoot proliferation. At 10°C, with zeatin (0.5 mg l^{-1}) in the medium, shoot proliferation was near zero (**Fig. 14**), (**Plate 9A**). This same result was also obtained when zeatin was substituted with either BA (1.5 mgl^{-1}) or kinetin (1.5 mgl^{-1}). But when cultures were incubated at 20°C, shoot proliferation activity increased sharply with either zeatin, BA, or kinetin in the medium (**Fig.14), (Plate 9B**). The optimal temperature for shoot proliferation activity with BA in the medium was 25°C. However, with zeatin or

kinetin in the medium, the number of shoots produced at 20°C and 25°C respectively were not statistically different (**Fig.14**). At 30°C, shoot proliferation activity was generally inhibited irrespective of the cytokinin in the basal medium. The few shoots produced at this temperature (30°C), were light green and shorter in length when compared to shoots obtained in cultures incubated at 20°C or 25°C (**Plate 9C**).

Maintaining *in vitro* cultures at a relatively high temperature reduced the efficacy of cytokinin which is basically responsible for shoot regeneration (GEORGE,1993). The few regenerated shoots at 30°C were shorter in length than shoots obtained in cultures incubated at 20°C and 25°C, and the leaves were light green. These results also showed that the temperature (30°C) which is above the determined optimal temperature for shoot regeneration was detrimental to shoot regeneration and growth. Previous workers have shown that temperature is one of the deciding factors for shoot multiplication and growth (FONNESBECH *et al.*, 1979; PIERIK *et al.*, 1988; HORN *et al.*, 1988; MEYER and VAN STADEN, 1991; PUDDEPHAT *et al.*, 1997).

2.3.4. Acclimatization

The results of various experiments carried out on rooting showed that rooting can best be achieved on MS (MURASHIGE and SKOOG, 1962) medium free of any plant growth regulator (**Plate 10A**). Plantlets commenced rooting within the first two weeks of incubation. Subsequently, rooted plantlets were planted into three different potting mixtures and were successfully acclimatized in the mist house for about four weeks before being transferred to the greenhouse (**Plate 10B**). Of all the potting mixtures employed, the highest survival, (98%) of plantlets, was obtained with a soil:sand:vermiculite mixture (1:1:1 v/v). It was also observed that mist house acclimatized *Aloe polyphylla* plants needed to be kept in at least 70 % shaded greenhouse for about six weeks to avoid direct sunlight at this early

Plate 10: (**A**) *Aloe polyphylla* rooting in plant growth regulator- free MS medium, (**B**) fully acclimatized *Aloe polyphylla* plants.

stage of development.

Results of this study showed that plantlets rooted well *in vitro* in a hormone-free medium. Generally, auxins are included in the medium at this stage for rooting, however there was no exogenous auxin in the medium and the plantlets rooted well. Previous studies on various species belonging to the Liliaceae showed that they can form roots in a hormone-free medium (NATALI *et al.*,1990; ROY and SARKAR, 1991; RICHWINE *et al.*, 1995). Since rooting is generally believed to be enhanced by auxins, it appears that the level of endogenous auxins in these species is high enough to promote rooting.

The higher survival percentage observed with the potting soil : sand : vermiculite (1: 1: 1 v/v) mixture when compared to peat, and potting soil respectively could mean that *Aloe polyphylla* survive better in a well aerated and drained soil mixture. Acclimatized plants (about eight months old) so far do not show any physical aberration.

2.3.5 Conclusion

At the end of this study, one could say that the cytokinins - kinetin , zeatin or BA alone enhanced shoot proliferation better than cytokinins and auxins in combination. In all optimal concentrations (cytokinin singly or in combination with auxin), both axillary and adventitious shoots were formed. Callus formation and hyperhydricity were very pronounced with BA, unlike the other cytokinins, and most shoots so formed with BA in the basal medium were stunted. Plantlets rooted better in a plant growth regulator- free MS medium than in MS with an auxin or in different strengths of MS medium (**Table 4**). The optimal temperature for shoot proliferation was observed at 25°C, though there was no significant difference with the number of shoots produced in cultures incubated at 20°C. Again, the optimal sucrose concentration for shoot proliferation was 3%. As for

potting mixture, the best for *in vitro* grown *Aloe polyphylla* was sand: soil: vermiculite (1:1:1 v/v). Mist house acclimatized plants need to be kept in at least a 70 % shaded greenhouse for about six weeks to avoid direct sunlight which tends to be harmful to the plants at this early stage of development.

Table 4: Mean number of roots produced by *Aloe polyphylla* at different strengths of MS medium.

MS medium	Mean no of Roots	No of replicates	Std. Deviation	Std. Error of Means
Full S	3.375	8	0.9161	0.3239
Half S	3.125	8	0.991	0.3504
One-quarter S	2.125	8	0.8345	0.295
Total	2.875	24	1.0347	0.2112

(S – Strength).

The clonal micropropagation method described here is very efficient in terms of both the rate and number of shoots produced and the rapidity at which new rooted shoots are obtained when compared with normal vegetative propagation. This protocol may prove to be a solution to the problem of declining numbers of *Aloe polyphylla* in nature.

Chapter Three

Tissue culture of *Platycerium bifurcatum*

3.1 Introduction

Tissue culture techniques have been employed not only in the propagation of *Platycerium bifurcatum,* but also in understanding its nutritional, physical and chemical requirements for growth and development. Previous tissue culture studies have focussed on the *in vitro* germination of spores (KNAUSS, 1976; BOURNE, 1994); the effects of nutrients and physical and chemical factors on growth and development of the prothallus, and on sporophyte formation (FERNANDEZ *et al.*, 1996; 1997) and the homogenization of gametophytes or sporophytes as an aid in sporophyte production (KNAUSS, 1976; COOKE, 1979; FINNIE and VAN STADEN, 1987; FERNANDEZ *et al.*, 1999).

3.1.1 Objectives

Platycerium bifurcatum is conventionally propagated sexually by spores and asexually through root bud development (RICHARDS *et al.*, 1983). According to Lane (1981), phytopathological problems are very common with *extra vitro* fern propagation from spores. Various workers have studied the *in vitro* propagation of *Platycerium bifurcatum*, but it seems that growth of the sporophyte *in vitro* is slow. It takes an average of six months to get to rooting size (JAMBOR, 1995). The strength of the MS medium has been shown to affect organogenesis and subsequent growth of plantlets *in vitro* (GEORGE, 1993). Therefore the objective of this part of the project was to investigate the effect of different strengths of the MS medium on organogenesis and subsequent growth of *Platycerium bifurcatum*, and different potting media on acclimatization.

3.2 Materials and methods

3.2.1 Decontamination procedures and aseptic techniques

Disinfection of plant materials is necessary in order to eradicate surface microorganisms. The presence of any contaminant will interfere with the growth of explants or cultures. Normally, prior washing of the explants with soap and water or dipping in ethanol is recommended to induce adequate wetting and initial cleaning. Most commonly a dilute solution of sodium hypochloride (0.25 - 2.63%) is used as a disinfectant, and an emulsifier such as Tween 20 is added at the rate of one drop per 100 ml solution. Vegetative explants are usually decontaminated for 10 - 20 minutes, followed by a rinse in autoclaved distilled water to remove residual disinfectant. Mechanical agitation using a stirrer is sometimes helpful to dislodge air bubbles and facilitate an even distribution of the disinfectant over the explant.

3.2.2 Explant source

The choice of a suitable explant is essential for successful tissue culture. The size, origin, and physiological status of an explant can also affect its response in culture. The explants used for this study were leaves of *Platycerium bifurcatum* collected from the Botanical Garden of the University of Natal (Pietermaritzburg). Leaves were rinsed under running water, soaked in 70% ethanol for a minute, washed in 1% NaOCl solution with a few drops of Tween 20 for ten minutes. Explants were afterwards rinsed three times in autoclaved distilled water. On completion of surface re-decontamination of the plant material, the explant was placed on a sterile petri dish for the removal of all the brown and discoloured areas. Leaves were cut into 1 cm squares. All further dissection took place on sterile petri dishes and the explants thus prepared were transferred to the culture vessels containing the nutrient medium.

3.2.3 Media and supplements

Leaf explants were placed on the basal medium supplemented with 1.0 mgl^{-1} of BA for adventitious bud proliferation.

In the subsequent experiment, after the initiation of adventitious buds, the effect of different strengths (full, half, and one-quarter strength) of the mineral salt constituents of the basal medium on the growth of plantlets was investigated.

3.2.4 Environmental conditions

Cultures were incubated at 25 ± 2°C with a 16 h light, 8 h dark photoperiod. Light was provided by cool white fluorescent tubes at flux density (400-700 nM) of 30 - 50 μmol m^{-2} s^{-1}.

3.2.5. Acclimatization

In vitro rooted plantlets of *Platycerium bifurcatum* were first washed in water to remove excess agar since sucrose and other organic compounds trapped by agar in the proximity of roots cause plantlets to be infected by disease, causing organisms to be damaged by toxic microbial metabolites (GEORGE, 1993). Plantlets were then potted in different planting mixtures - peat; 1 peat: 1 vermiculite: 1 sand. Plants were kept in the mist house (at a temperature of 24°C ± 2°C) for six weeks, after which they were transferred to the green house.

3.3 Results and Discussion

3.3.1 Decontamination procedures

The decontamination procedure -soaking explants in 70 % ethanol for a minute, and afterwards washing explants in 1 % NaOCl solution with a few drops of Tween 20 described in 3.2.1 was very successful. Almost 80 % of the explants were decontaminated. Due to the success achieved with this decontamination procedure no further effort was made to try other decontamination procedures.

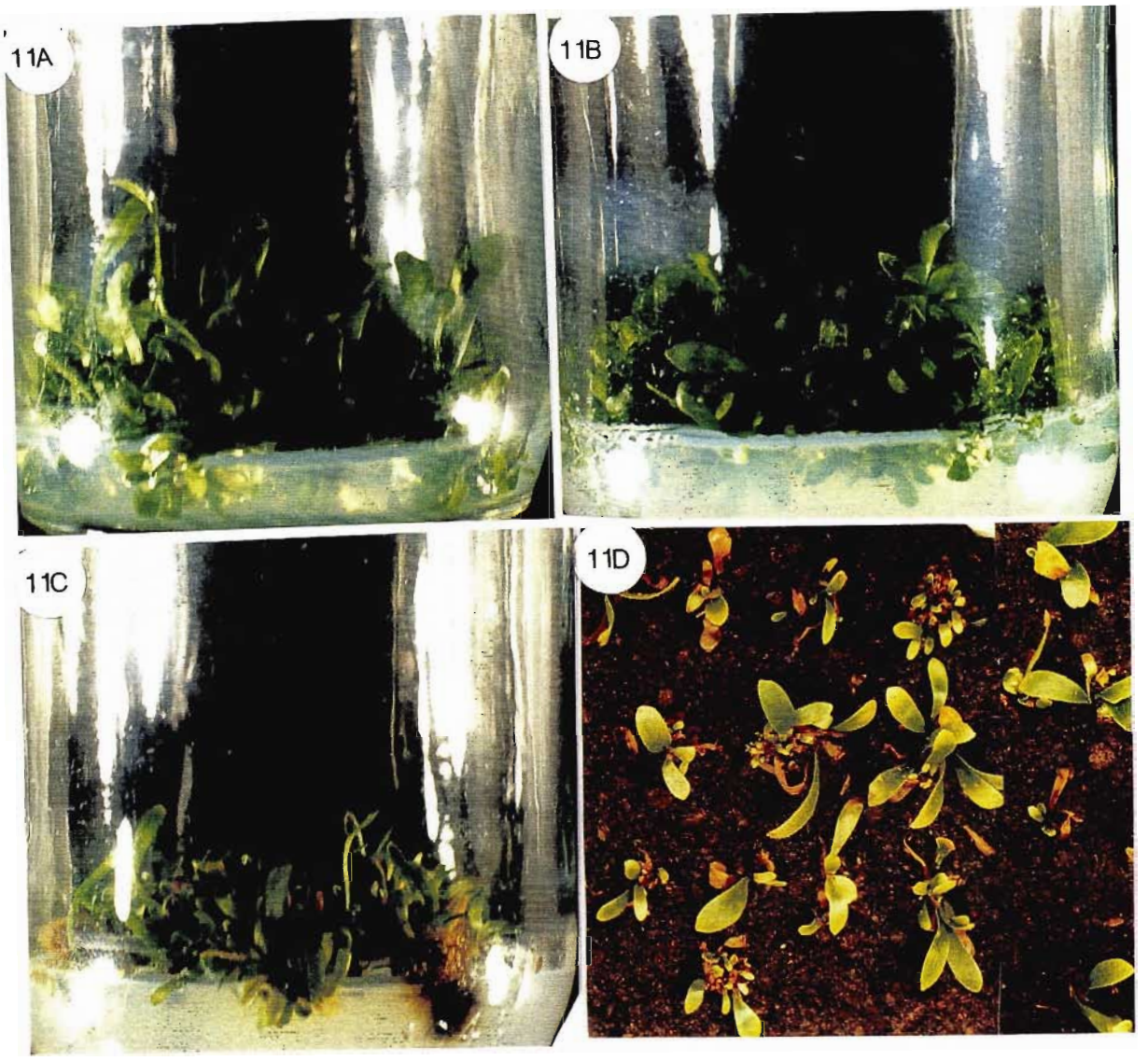


Plate 11: (**A**) *Platycerium bifurcatum* cultured in half-strength MS medium, (**B**) in full strength MS medium, (**C**) one-quarter strength MS medium, (**D**) fully acclimatized plants.

3.3.2 Media and supplements

For this part of the project, the number of proliferating buds was not recorded since it was not the focus of the experiment. The focus of the experiment was the growth and subsequent development of the plantlets. The results are therefore presented in visual form only.

Adventitious buds appeared on the leaf surface without callus formation in the basal medium supplemented with 1.0 mg l^{-1} BA. After adventitious bud initiation, further bud proliferation was observed on full strength and half-strength MS media. Adventitious buds were isolated and subcultured monthly on different strengths of MS media (full strength, half-strength, and one-quarter strength MS media). On half-strength MS medium, bud growth was very vigorous (**Plate 11A**), and plantlets rooted well in this medium. With full strength MS medium, bud growth was observed **(Plate 11B)** although it was not as pronounced as bud growth observed in half-strength MS medium. There was root initiation in this medium, but fewer roots were present compared to the half-strength MS medium treatment. When buds were subcultured in one-quarter strength MS medium, bud growth was the slowest. The leaves were yellow **(Plate 11C)**. Root development was the best in this medium.

Results showed that after bud initiation on a growth regulator containing MS medium, further growth and development of buds can best be achieved in half-strength MS medium without growth regulators. Further proliferation of buds observed after subculturing on a plant growth regulator - free MS medium could be attributed to either a carry-over effect of BA, or the ability of explants to proliferate buds in a plant growth regulator - free medium, as reported by previous workers (CAMLOH and GOGALA, 1991; CAMLOHA *et al.*, 1993; BOURNE, 1994). Generally, research has shown that organogenesis is plant growth regulator-controlled. However, the subsequent organogenesis of buds

without exogenous growth regulators might reflect high endogenous growth regulator levels.

3.3.3 Acclimatization

The strength of the MS medium played a major role in the rooting of *Platycerium bifurcatum in vitro*. Plantlets subcultured on one-quarter strength of MS medium rooted better than plantlets subcultured on the other two media (full strength and half-strength MS respectively). This result was obtained without any plant growth regulator in the medium. This suggests that the plantlets might have had sufficient endogenous auxins to initiate rooting. Results obtained are similar to that of Gleba and Gordzievskaya (1987), while working on *in vitro* propagation of *Platycerium bifurcatum*. They also successfully achieved rooting in MS medium without plant growth regulators and in MS medium diluted ten-fold.

Rooted plantlets of about 3-4 cm in height were isolated and planted into two different potting mixtures (peat; peat: vermiculite: sand (1: 1: 1) v/v). They were acclimatized in the mist house for about six weeks before being transferred to the greenhouse (**Plate 11D**). Highest survival (90 %) of plantlets was obtained with peat alone (**Table 5**). By comparing the water retaining ability of the two different potting mixtures, it is clear that *Platycerium bifurcatum* thrived better on a high water retaining potting mixture. Successfully acclimatized plants are doing well in the greenhouse.

Table 5: Percentage survival of *in vitro* grown *Platycerium bifurcatum* planted out on two different potting mixtures

Potting mixture	No. of transplanted plants	No. of survived plants	Survival (%)
Peat	40	36	90
Peat:Vermiculite: sand (1: 1: l v/v)	40	22	56

3.3.4 Conclusion

Platycerium bifurcatum showed a high multiplication rate using leaf explants. The subsequent isolation and subculturing of plantlets were, however, labour intensive. Although growth of plantlets in half-strength MS medium was satisfactory, rooting was better in one-quarter strength MS than the half-strength MS medium. To ensure better rooting of plantlets, it is best to monthly isolate plantlets from half-strength MS medium and then subculture them on one-quarter strength medium for about five weeks.

Peat proved a better potting mixture in terms of plantlet survival.

Chapter Four

General Conclusion

Having presented the results of this study in Chapters two and three, it is necessary to briefly conclude the salient points of this investigation. Many rare and endangered plant species are propagated *in vitro* because they do not respond well to conventional methods of propagation. As far as I know there is only one report on tissue culture of *Aloe polyphylla* (ABRIE and VAN STADEN, 2001) despite its spectacular ornamental and 'medicinal' values. An optimized tissue culture protocol was developed to produce a large number of plants of this rare species of *Aloe*. Shoot explants derived from *in vitro* grown plantlets were inoculated onto MURASHIGE SKOOG (MS) (1962) medium supplemented with 100 mg l^{-1} myo-inositol, 3 % sucrose, 0.8 % agar, and different concentrations of various cytokinins singly or in combination with the auxins naphthaleactic acid (NAA) or indole-butryic acid (IBA). These explants produced multiple shoots. The average number of plantlets produced per explant was high, ranging from six to ten. There was a yellowish callus formed at the base of the explants especially when 3.0 mg l^{-1} of the cytokinins (kinetin, iP and zeatin) were used. Hyperhydricity was encountered with BA in the multiplication medium. Hyperhydricity with BA needs further research because of the fact that BA induced the highest average number of plantlets.

Sucrose and temperature proved to be amongst the most important determining factors in shoot proliferation. Sucrose at the 3 % level and temperature at 25°C were ideal for shoot proliferation. Plantlets were successfully rooted in a hormone-free MS medium and subsequently acclimatized. This protocol is assumed to be effective considering the high regeneration percentage of explants and also the high number of plantlets produced. The ease with which rooting was achieved without any auxin in the medium makes the protocol desirable.

Plantlets cultured *in vitro* are highly susceptible to dessication once transferred to soil. Survival of plants depends on their ability to carry out photosynthesis and withstand water loss. *Aloe polyphylla* showed a good ability to carry out photosynthesis and withstand water loss. This is reflected in the high survival rates (almost 100 %). *Aloe polyphylla* remains an endangered species. However, the future of this species is now less precarious due to the success of the multiplication protocol now available.

In the second investigation, using leaf explants, *Platycerium bifurcatum* showed a high multiplication rate. Growth and rooting of plantlets were best achieved in half- and one-quarter strength MURASHIGE and SKOOG (MS) (1962) media, each supplemented with 100 mg l^{-1} myo-inositol, 3 % sucrose, and 0.8 % agar. The growth of plantlets was very satisfactory. Plantlets were successfully acclimatized. The major problem to be solved is the labour intense monthly subculturing of the plantlets. This problem must be addressed in the future.

References

ABRIE AL and VAN STADEN J (2001) Micropropagation of the endangered *Aloe polyphylla*. Plant Growth Regulation **33**: 19-23.

ALTMAN A and ZIV M (1997) Horticultural biotechnology: A historical perspective and future prospects. *Acta Horticulturae* **447**: 31-35.

AMBROZIC D and CAMLOH M (1997) Gametophytic and sporophytic regeneration of *Platycerium. Annals of Botany* **80**: 23-28.

BINDING H and KRUMBIEGEL-SCHWEREN G (1984) Clonal propagation: shoot cultures. Cell Culture and Somatic Cell Genetic of Plants, Vol. 1, 43-48.

BOURNE R A (1994) The development of a program of commercial production of staghorns from plant tissue culture. *Combined Proceedings International Plant propagators' Society* **44**: 90-93.

BRAINERD KE and FUCHIGAMI LH (1981) Acclimatization of aseptically cultured Apple plants to low relative humidity. *Journal of American Society of Horticultural Science* **106**: 515-518

CAMLOH M, RAVNIKAR M and ZEL J (1996) Jasmonic acid promotes division of fern protoplasts, elongation rhizoids and early development of gametophytes. *Physiologia Plantarum* **97**: 659-664.

CAMLOH M, VILHAR B, ZEL J and RAVNIKAR M (1999) Jasmonic acid stimulates development of rhizoids and shoots in fern leaf culture. *Journal of Plant Physiology* **155**: 798-801.

CAMLOHA M and GOGALA N (1991) *Platycerium bifurcatum* – adventitious bud and root formation without growth regulators *in vitro*. *Acta Horticulturae* **289** :89-90.

CAMLOHA M and GOGALA N (1992) *In vitro* culture of *Platycerium bifurcatum* gametophytes. *Scientia Horticulturae* **51**: 343-346.

CAMLOHA M, GOGALA N and RODE J (1994) Plant regeneration from leaf explants of the fern *Platycerium bifurcatum in vitro. Scientia Horticulturae* **56** :257-266.

CASTORENA SANCHEZ I, NATALI L and CAVALLINI A (1988) *In vitro* culture of *Aloe barbadensis*: morphogenetic ability and nuclear DNA content. *Plant Science*. **55**:53-59.

CAVALLINI A, NATALI L, CIONINI G, SASSOLI O and CASTORENA-SANCHEZ I (1993) *In vitro* culture of *Aloe barbadensis*: quantitative DNA variation in regenerated plants. *Plant Science Limerick* **91**: 223-229.

CHIN WY (1997) Ferns of the tropics. Times editions, Singapore.

COOKE RC (1979) Homogenization as an aid in tissue culture propagation of *Platycerium* and *Davallia. HortScience* **14**: 21-22.

CORNEANU M, CORNEANU G, BADICA C, MINEA R, BICA D and VEKAS L (1994) *In vitro* organogenesis of *Aloe aborescens. Revue Roumaine de Biologie Vegetale* **39**: 45-52.

DE FOSSARD RA (1981) Plant Tissue Culture propagation. Filmfiche Corporation, Sydney.

DEBERGH P, ROGGEMANS J and STANDAERT-DEMETSENAERE R (1990) Tissue culture in relation to ornamental plants. *In*: Plant Tissue Culture: Applications and Limitations. (Ed) Bhojwani SS, Elsevier, Amsterdam. Pp 161-189.

DEBERGH P (1994) *In vitro* culture of ornamentals: *In*: Plant Cell and Tissue Culture. (Eds) Vasil KI and Thorpe TA, Kluwer Academic Publishers, Dordrecht, Netherlands. Pp 561-572.

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

DUNSTAN DI and TURNER KE (1984) The acclimatization of micropropagated plants: *In:* Cell Culture and Somatic Genetics of Plants. (Ed) Vasil IK, vol.1 Laboratory Procedures and their Applications, Academic Press, Inc., Orlando, Florida.

EMANOIL M (1994) Encyclopedia of Endangered Species. Gale Research inc., Michigan. Pp 1091-1092.

FENG F, LI HB, LU QF and XIE JY (2000) Tissue Culture of *Aloe* spp. *Journal of Southwest Agricultural University* **22**: 157-159.

FERNANDEZ H, BERTRAND AM and SANCHEZ-TAMES R (1999) Biological and nutritional aspects involved in fern multiplication. *Plant Cell, Tissue and Organ Culture* **56**:211-214.

FERNANDEZ H, BERTRAND AM and SANCHEZ-TAMES R (1996) Influence of tissue culture conditions on apogamy in *Dryopteris affinis*. *Plant Cell, Tissue and Organ Culture* **45**: 93-97.

FERNANDEZ H, BERTRAND AM and SANCHEZ-TAMES R (1997) Germination in cultured gametophytes of *Osmunda regalis*. *Plant Cell Report* **16**: 358-362.

FINNIE JF and VAN STADEN J (1987) Multiplication of the tree fern *Cyathea dregei*. *Horticultural science* **22**: 665.

FONNESBECH A, FONNESBECH M and BREDMOSE N (1979) Influence of cytokinins and temperature on development of *Asporagus plumosis* shoot tip *in vitro*. *Physiology Plant* **45**: 73- 76.

GEORGE EF (1993) Plant Propagation by Tissue Culture: Part 1: The Technology, 2nd edition, Exegetics Ltd., Everseley.

GLEBA DM and GORDZIEVSKAYA LP (1987) Propagation of *Platycerium bifurcatum* in *in vitro* culture. *Introducktsiya i Rastenii* **7**: 59-61.

GRESSHOF PM (1978) Phytohormones and Growth and Differentiation of Cells and Tissues Cultured *in vitro*. *In*: Phytohormones and Related Compounds - A Comprehensive Treatise. Vol II. Phytohormones and the Development of Higher Plants. (Eds) Letham DS, Godwin PB and Higgins JJV. Elsevier/North Holland Biomedical Press, Amsterdam, Oxford, New York. Pp 1-22.

GROENEWALD EG, KOELEMAN A and WESSELS DCJ (1979) The use of tissue cultures in the propagation and possible hybridization of *Aloes* and related plants. *Aloe* **17**: 37-40

GROENEWALD EG, KOELEMAN A and WESSELS DCJ (1975) Callus formation and plant regeneration from seed tissue of *Aloe pretoriensis*. *Zeitschift Jur Pflangenphysiology* **75:** 270-272.

GROENEWALD EG, WESSELS DCJ and KOELEMAN A (1976) Embryoid formation in callus culture of *Aloe pretoriensis*. *South African Journal of Science* **72**: 89-90.

GUI YL, XU TY, GU SR, LIU SQ, ZHANG Z, SUN GD and ZHANG Q (1990) Studies on stem tissue culture and organogenesis of *Aloe vera*. *Acta Botanica Sinica* **32**: 606- 610.

HARTMANN HT and KESTER DE (1983) Plant Propagation: Principles and Practices. Prentice - Hall, Englewood Cliffs, New Jersey. Pp 53-149.

HAYASHI M (1987) Callus characteristics and classification of *Haworthia* and allied genera. South African Journal of Botany **53**: 411-423.

HENNEN GR and SHEEHAN TJ (1978) *In vitro* propagation of *Platycerium stemaria*. *HortScience* **13**: 245.

HENNIPMAN E and ROOS MC (1982) A Monograph of the Fern Genus *Platycerium* (Polypodiaceae). North Holland Publishing Company, Amsterdam.

HICKOK LG, WARNE TR and SLOCUM MK (1987) *Ceratopteris richardii:* Applications for experimental plant biology. *American Journal of Botany* **74**: 1303-1316.

HIGUCHI H, AMAKI W and SUZUKI S (1987) *In vitro* propagation of *Nephrolepis cordifolia*. *Presl. Scientia Horticulturae* **32**: 105-113.

HIRIMBUREGAMA K and GAMAGE N (1995) *In vitro* multiplication of *Aloe vera* meristem tips for mass propagation. *Horticultural Science* **27**: 15-18.

HORN W, SCHLEGEL G and KARIN J (1988) Micropropagation of roses (Rosa hybr.). *Acta Horticulturae* **226**: 623-625.

HOSHIZAKI JH (1972) Morphology and phylogeny of *Platycerium* species. *Biotropica* **4**: 93-117.

HUSSEY G (1979) Tissue culture and its application to plant propagation. *Plantsman* **1**: 133-145.

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

JAMBOR BE, CSILLAG A, MARTA RA and CSIZMADIA G (1995) *In vitro* regeneration and propagation of *Platycerium bifurcatum*. *Acta Agronomica Hungarica* **43**: 1-2.

KAWAI K, BEPPU H, KOIKE T, FUJITA K and MARUNOUCHI T (1993) Tissue culture of *Aloe arborescens*. International Congress of Phytotherapy. *Phytotherapy Research* **7**: 5-10.

KIM JK, HWANG IT and KIM HE (1996) Micropropagation by *in vitro* spore culture and potting medium in *Platycerium bifurcatum*. *RDA Journal of Agricultural Science - Biotechnology* **38**: 267-272.

KNAUSS JF (1976) A partial tissue culture method for a pathogen- free propagation of selected ferns from spores. *Proceedings of Florida State Horticultural Society* **89**: 363-365.

KOOPOWITZ H and KAYE H (1990) Plant Extinction: A Global Crisis. (Ed.) Knees S, Christopher Helm, London. Pp 190-191.

KWA SH, WEE YC, LIM TM and KUMAR PP (1997) Morphogenetic plasticity of callus reinitiated from cell suspension cultures of the fern *Platycerium coronarium*. *Plant Cell, Tissue and Organ Culture* **48**: 37-44.

KWA SH, WEE YC and KUMAR PP (1995) Role of ethylene in the production of sporophytes from *Platycerium coronarium* (Koenig) Desv. Frond and rhizome pieces cultured *in vitro*. *Journal of Plant Growth Regulation* **14**:183-189.

LANE BC (1981) A procedure for propagating ferns from spores using a nutrient-agar solution. *Combined Proceedings of International Plant Propagators Society* **30**: 94-97.

LANGFORD PJ and WAINWRIGHT H (1988) Influence of sucrose concentration on the photosynthetic ability of *in vitro* grown rose shoots. *Acta Horticulturae* **227**: 305-307.

LESHAM B, WERKER E and SHALEV DP (1988) The effect of cytokinins on vitrification in melon and carnation. *Annals of Botany* **62**: 271-276.

LI Y, WANG LH, YE MM and SHEN DL (1997) The factors influencing vitrification of tissue-cultured carnation plantlets. *Plant Physiology Communications* **33**: 256-258.

LINSMAIER EM and SKOOG F (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum* **18**: 100-125.

MELAN MA and WHITTIER DP (1990) Effects of inorganic nitrogen sources on spore germination and gametophyte growth in *Botrychium dissectum. Plant Cell Envir.* **13**: 477-482.

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

MILLER JH and WAGNER PM (1987) Co-requirement of calcium and potassium in the germination of spores of the fern *Onoclea sensibilis. American Journal of Botany* **74**: 1585-1589.

MOREL GM and MARTIN G (1952) Guerison de *Dahlia* atteints d` une maladie a virus. C. R. lebd. Seance. *Acad. Sci. Paris* **235**: 1324-1325.

MURASHIGE T (1974) Plant propagation through tissue culture. *Annual Review of Plant Physiology* **25**:135-165.

MURASHIGE T (1976) Clonal Crops through Tissue Culture. In: Proceedings in life sciences: Plant tissue culture and its biotechnological applications. (Eds) Barz W, Reinhard E and Zenk MH, Springer-Verlag, Berlin. Pp 393-403.

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

NATALI L, SANCHEZ IC and CAVALLINI A (1990) *In vitro* culture of *Aloe barbadensis*: micropropagation from vegetative meristems. *Plant Cell, Tissue and Organ Culture* **20**:71-74.

NESTER JE and COOLBAUGH RC (1986) Factors influencing spore germination and early gametophyte development in *Anemia mexicana* and *Anemia phyllitidis. Plant Physiology* **82**: 230-235.

NITSCH C and NITSCH JP (1967) The induction of flowering *in vitro* in stem segments of *Plumbago indica* L. *Planta* **72**: 355-370.

PADHYA MA and MEHTA AR (1982) Propagation of fern (*Nephrolepis*) through tissue culture. *Plant Cell Reports* **1**: 261-263.

PEVALEK KB (1996) Effect of sucrose and agar concentration, and medium pH on staghorn fern (*Platycerium bifurcatum* (Chr.) C. Cav.) Shoot multiplication. *Horticultural Science* **28**: 3-4, 18-20.

PIERIK RLM, STEEGMANS HHM, ELIAS AA, STIEKEMA OTJ and VAN DER VELDE AJ (1988) Vegetative propagation of *Syringa vulgaris* L. *in vitro. Acta Horticulturae* **226**: 195-101.

PUDDEPHAT J, ALDERSON PG and WRIGHT NA (1997) Influence of explant source, plant growth regulators and culture environment on culture initiation and establishment of *Quercus robur* L. *in vitro. Journal of Experimental Botany* **48 (309)**: 951-962.

RACCHI ML (1988) Using *in vitro* culture to study the biosynthesis of secondary products in *Aloe ferox. Rivista di Agriculture Subtropicale e Tropicale* **82(4)**: 707-714.

REUTHER G (1983) Propagation of disease-free *Pelargonium* cultivars by tissue culture. *Acta Horticulturae* **131**: 311 - 319.

RICHARDS JH, BECK Z and HIRSCH AM (1983) Structural investigations of asexual reproduction in *Nephrolepsis exaltata* and *Platycerium bifurcatum. American Journal of Botany* **70**: 993-1001.

RICHWINE AM, TIPTON JL and THOMPSON GA (1995) Establishment of *Aloe, Gasteria,* and *Haworthia* shoot cultures from inflorescence explants. *HortScience* **30**: 1443-1444.

ROY SC and SARKAR A (1991) *In vitro* regeneration and micropropagation of *Aloe vera. Scientia Horticulturae* **47**:107-113.

SCHWANN T (1839) Mikrocopische Untersuchungen uber die U bereinstimmung in der Thiere und Pflanzen, Oswalds, Berlin.

SHARP WR and LARSEN PO (1979) Plant Cell and Tissue Culture: Current applications and potentials. *In*: Plant Cell and Tissue Culture. Principles and Applications, ed. Sharp, W. R., Larsen, P. O., Paddock, E. F. and Raghanvan, V., Ohio state university, Columbus. Pp 115-120.

SPIEGEL-ROY P and KOCHBA J (1977) Application of Tissue Culture for Plant Improvement: *In*: Proceedings in Life Sciences, Plant Tissue Culture and its Bio-technological Applications. (Eds) Barz W, Reinhard E and Zenk MH, Springer-Verlag, New York. Pp 404-411.

SUTTER E and LANGHANS RW (1979) Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. *Journal of American Society of Horticultural Sciences* **104**: 493-496

THENTZ M and MONCOUSIN C (1984) *In vitro* micropropagation of *Platycerium bifurcatum. Revue Horticole Suisse* **57**: 293-297.

THOMAS P, MYTHILI JB and SHIVASHANKARA KS (2000) Explant, medium and vessel aeration affect the incidence of hyperhydricity and recovery of normal plantlets in triploid watermelon. *Journal of Horticultural Science and Biotechnology* **75**:19-25.

THORPE TA and MEIER DD (1972) Starch Metabolism, Respiration, and Shoot Formation in Tobacco callus Culture. *Physiologia Plantarum* **27**: 365-369.

THORPE TA and MURASHIGE T (1968) Starch accumulation in shoot forming tobacco callus cultures. *Science* **160**: 421-422.

THORPE TA and MURASHIGE T (1970) Some histochemical changes underlying shoot initiation in tobacco callus cultures. *Canadian Journal of Botany* **48**: 277-285.

TSAY HS and DREW RA (1998) Effect of medium compositions at different recultures on vitrification of carnation (*Dianthus caryophyllus*) *in vitro* shoot proliferation. *Acta Horticulturae* **461**: 243-249.

VAN WYK BE, VAN OUDTSHOORN B and GERICKE N (1997) Medicinal plants of South Africa. Briza publications, Pretoria.

VAN WYK B and SMITH G (1996) Guide to the *Aloes* of South Africa. Briza publications, Pretoria. Pp 154-155.

VASIL IK and VASIL V (1980) Clonal propagation. *International Review of Cytology* **11A**: 145-173.

VIJ SP, SHARMA M and TOOR IS (1980) Cytogenetical investigation into some garden ornamentals II. The genus *Aloe*. *Cytologia* **45**:515-532.

WETZSTEIN HY and SOMMER HE (1982) Leaf anatomy of tissue-cultured *Liquidambar stryaciflua* (Hammelidaceae) during acclimatization. *American Journal of Botany* **69**: 1579-1586

YEOMAN MM (1973) Tissue (callus) cultures - techniques. *In*: Plant tissue and cell culture. Botanical monographs. Vol. II. (Ed) Street HE, Blackwell scientific publications, Oxford. Pp 31-58.

YEOMAN MM and AITCHISON PA (1973) Growth patterns in tissue (callus) cultures. *In*: Plant tissue and cell culture. Botanical monographs. Vol. II. (Ed) Street HE, Blackwell scientific publications, Oxford. Pp 240-267.

ZHAO M (1990) Rapid propagation of *Aloe variegata* by tissue culture. *Journal of Shenyang Agricultural University* **21**: 339-340.

ZHOU GY, DING HF, SHI WM and CHENG L (1999) Fast asexual propagation of *Aloe vera*. *Acta Horticulturae Sinica* **26**: 410-411.