CULTIVATION OF *COMBRETUM BRACTEOSUM* (HOCHST.) BRANDIS

by

Kerry Jacqueline Koen

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PREFACE

The experimental work described in this dissertation was conducted in the School of Botany and Zoology, University of Natal, Pietermaritzburg, from 1998 to 2001 under the supervision of Professor J. van Staden.

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

KJKoen

KERRY JACQUELINE KOEN

OCTOBER 2001

I certify that the above statement is correct.

J. Staden

PROF. J. VAN STADEN

(SUPERVISOR)

PUBLICATION

The following publication was produced during the course of this study:

1. DALLING, K.J. and VAN STADEN, J. 1999. Germination requirements of *Combretum bracteosum* seeds. *South African Journal of Botany* **65:** 83-85.

PAPERS PRESENTED AT SCIENTIFIC CONFERENCES

1. DALLING, K. and VAN STADEN, J. 1998. Propagation of *Combretum bracteosum*. 25th Annual Congress of SAAB, University of Transkei, Umtata.

2. DALLING, K. and VAN STADEN, J. 1999. *Combretum bracteosum:* A striking ornamental shrub, now more suitable for smaller gardens. 26th Annual Congress of SAAB, Potchefstroom University for Christian Higher Education.

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ABSTRACT

In maximizing South Africa's floral diversity, plant propagators have begun exploiting the rich array of indigenous plants, especially those with horticultural potential. Plants previously unavailable to the professional and amateur gardeners alike, are legally becoming common-place in nurseries. However, in promoting the trade of indigenous plants to nursery-owners, rapid, easy and cost effective methods of propagating these plants need to be established.

Combretum bracteosum is one such indigenous plant, the aesthetic appeal thereof exhibits great potential for ornamentation, especially when flowering. In facilitating the introduction of *Combretum bracteosum* into nurseries, small gardens or even pots, investigations carried out aimed to determine and analyse quick and easy methods of propagating this plant. Of the various propagation techniques considered, only one, micropropagation, required specialized skill and training prior to carrying out the relevant procedures and protocols. The two other techniques used, which are accessible to most plant propagators, were seed germination and propagation from cuttings.

Propagation by seed germination yielded less than optimal results from a commercial perspective. Although the hard pericarp surrounding the embryo did not impose any dormancy inducing mechanisms, such as the restriction of water uptake or the leaching of an inhibitory compounds, it did act as a mechanical barrier to the emerging radicle and roots. Recommendations for optimal *Combretum bracteosum* seed germination would be to remove the protective pericarp completely, incubate imbibed embryos in complete darkness at 25 °C. After radicle emergence the germinating embryos could be moved into an alternating light: dark cycle.

A more viable and simpler alternative to seed germination, was propagation by stem cuttings. Treating the cuttings with 10% and 50% or 75% of the commercially available

Kelpak concentrate (using the Soak Method and Quick-dip Methods respectively), provided the most promising results, with the rapid development of roots and subsequent vegetative growth. Synthetic hormones such as IBA and NAA were also applied to the cuttings both alone or in combination however, although callus growth was profuse, root development was slow and unsubstantial, if any at all. Therefore, in recommending a protocol for the successful rooting of *Combretum bracteosum* cuttings taken during spring, summer or early autumn, the application of Kelpak at either 10% (Soak Method) or 50% (Quick-dip Method) of the full strength solution, is advised. Subsequent to hormone treatment, the cuttings still required attention with regard to nutrient supplementation as well as atmospheric moisture and temperature regulation.

Success in generating *Combretum bracteosum* plantlets was obtained by germinating the seed *in vitro* as well as stimulating axillary shoot elongation from nodal explants. Placing the sterilized *Combretum bracteosum* embryo onto a nutrient rich basal medium (containing no hormones) was sufficient to stimulate 100% germination. The frequent poor availability of the seed may hamper the use of *in vitro* seed germination for commercial propagation purposes. The use of nodal explants from *in vitro* germinated stock plants, is a rapid and reliable means of generating a large seedling stock. Nodal explants excised from the newly developed shoot were subsequently placed onto 0.5 $mg.\ell^{-1}$ BA which encouraged axillary bud elongation. After elongation, the lateral shoots were removed and placed onto a rooting medium (1.0 $mg.\ell^{-1}$ IBA).

The more mature nodal explants, collected from parent plants growing *in vivo*, required either a BA: NAA hormone combination or Kelpak to stimulate axillary shoot elongation, with the latter being most successful. Root initiation followed the protocol described above. Once rooted plantlets were hardened off they displayed a strong and vigorous growth, which is desirable from a commercial perspective.

Upon maturity, the habit of many indigenous trees and shrubs could become too big for

confined spaces such as the urban garden. Therefore, determining a means of modifying the plants' habit in order to maintain its suitability as a smaller garden plant was important. Treating the *Combretum bracteosum* plants with a 50 $mg.\ell^{-1}$ paclobutrazol soil drench proved most successful, with the desired effects being visible within a few weeks of initial application. No negative morphological or developmental effects were noted on plants treated with the dwarfing agent, conversely however, the treated *Combretum bracteosum* plants were compact and bushy, with considerable visual appeal and aesthetic attractiveness.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
ANOVA	Analysis of variance
±	approximately
BA	Benzyladenine
C	carbon
°C	degrees Centigrade
Ca	calcium
cm	centimeters
CO_2	carbon dioxide
°	degrees
DMF	N,N-dimethylformamide
E	east
Fe	iron
g	grams
$g.\ell^{-1}$	grams per liter
GA_3	Gibberellin A_3; gibberellic acid
GA_7	Gibberellin A_7
▸	greater than
≥	greater than or equal to
$HgCl_2$	mecuric chloride
IAA	indole-3-acetic acid
IAAox	indole-3-acetic acid oxidase
IBA	indolebutyric acid
K	potassium
KIN	kinetin
LSD	Least Significant Difference
′	minutes

″	seconds
m	meters
M	moles
Mg	magnesium
$mg.\ell^{-1}$	milligram per liter
ml	millilitres
mm	millimeters
Mn	manganese
MS	MURASHIGE and SKOOG (1962) nutrient medium
N	nitrogen
NAA	∝-Napthaleneacetic acid
NaOCl	sodium hyperchlorite
nm	nanometers
O_2	oxygen
%	percent
P	phosphorus
PVP	polyvinylpolypyrrolidone
R:FR	Red: Far Red
PGR	Plant Growth Regulators
PP333	Paclobutrazol
S	south
S.E.	Standard Error
SWC	seaweed concentrate
WPM	Woody Plant Nutrient Medium
Zn	zinc

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This thesis is dedicated to my parents, who have sacrificed so much to put me through university and my husband Pete, on whose love and support I have relied

Chapter 1
General Introduction

It is universally accepted that Southern Africa has been favoured by nature in many ways. Among her assets is a rich indigenous flora - rich in the variety of species and rich in their floral beauty. As South Africans is there a need to look further than our own backyard in order to beautify our gardens and open spaces in urban areas? Although propagation is looked on by many people as a rather advanced skill (and certainly there are some plant species where propagation calls for great skill and sensitivity), there are a large number of plants which are not at all difficult to propagate successfully. It is therefore our responsibility to investigate different propagation methods in an attempt to bring indigenous flora into our communities and gardens, and thus optimise the full potential of these exquisite plants.

Combretum bracteosum (Hochst.) Brandis, belongs to the family Combretaceae (bushwillow, white mangrove). Belonging to the order Myrtales, the Combretaceae (Figure 1) are divided into two sub-families of which only the Combretiodeae is of present concern. Of the two tribes comprising this sub-family, it is the Combreteae, (further divided into three sub-tribes) which is of significance in Africa. The genus from the sub-tribe (Combretinae), important in this investigation is *Combretum*. The family and gender name Combretaceae or *Combretum* is said to originate from the Roman naturalist Pliny (the Elder), who gave the name to a certain species of climber, today unknown (CARR, 1988). According to ROGERS and VEROTTA (1996), the African Combretaceae is represented by twenty genera, with about 480 species of trees, shrubs, climbers (sometimes spinose) and a mangrove. These are all found in the tropical and subtropical parts of the continent. Other than in the extreme southern and south western areas of South Africa, its members are widespread within this country. The plants are characterised by their spicate inflorescence and fruits with winged-shaped appendages. Eight genera of the Combretaceae are found in southern Africa. The largest of these is *Combretum*, with approximately 250

Order: **MYRTALES**

Family: COMBRETACEAE

Sub-families (20 genera):

COMBRETOIDEAE
19 Genera

STREHONEMATOIDEAE
1 Genus: *Strephonema* (7 spp.)

Tribes:

COMBRETEAE
16 Genera

LAGUNCULARIEAE
3 Genera: Lagucularia (2spp.)
Lumnitzera (2 spp.)
Macropteranthus (4 spp.)

Sub-tribes:

COMBRETINAE
7 Genera

PTELEOPSIDINAE
1 Genus

TERMINALINAE
8 Genera

Genera:

1. *Thiloa* (3 spp.)
2. *Quisqualis* (16 spp.)
3. *Calopyxis* (22 spp.)
4. *Meiostemon* (2 spp.)
5. *Guiera* (1 spp.)
6. *Calycopteris* (1 spp.)
7. ***Combretum* (± 200 spp.)**

1. *Pteleopsis* (10 spp.)

1. *Terminalia* (± 150 spp.)
2. *Ramatuella* (6 spp.)
3. *Terminaliopsis* (1 spp.)
4. *Bucida* (9 spp.)
5. *Buchenavia* (22 spp.)
6. *Anogeissus* (14 spp.)
7. *Finetia* (1 spp.)
8. *Conocarpus* (12 spp.)

Sub-genus: Combretum (31 sections) Cacoucia (13 Sections) Apetalanthum (1 section)

Figure 1: Taxonomic Classification of the Family Combretaceae

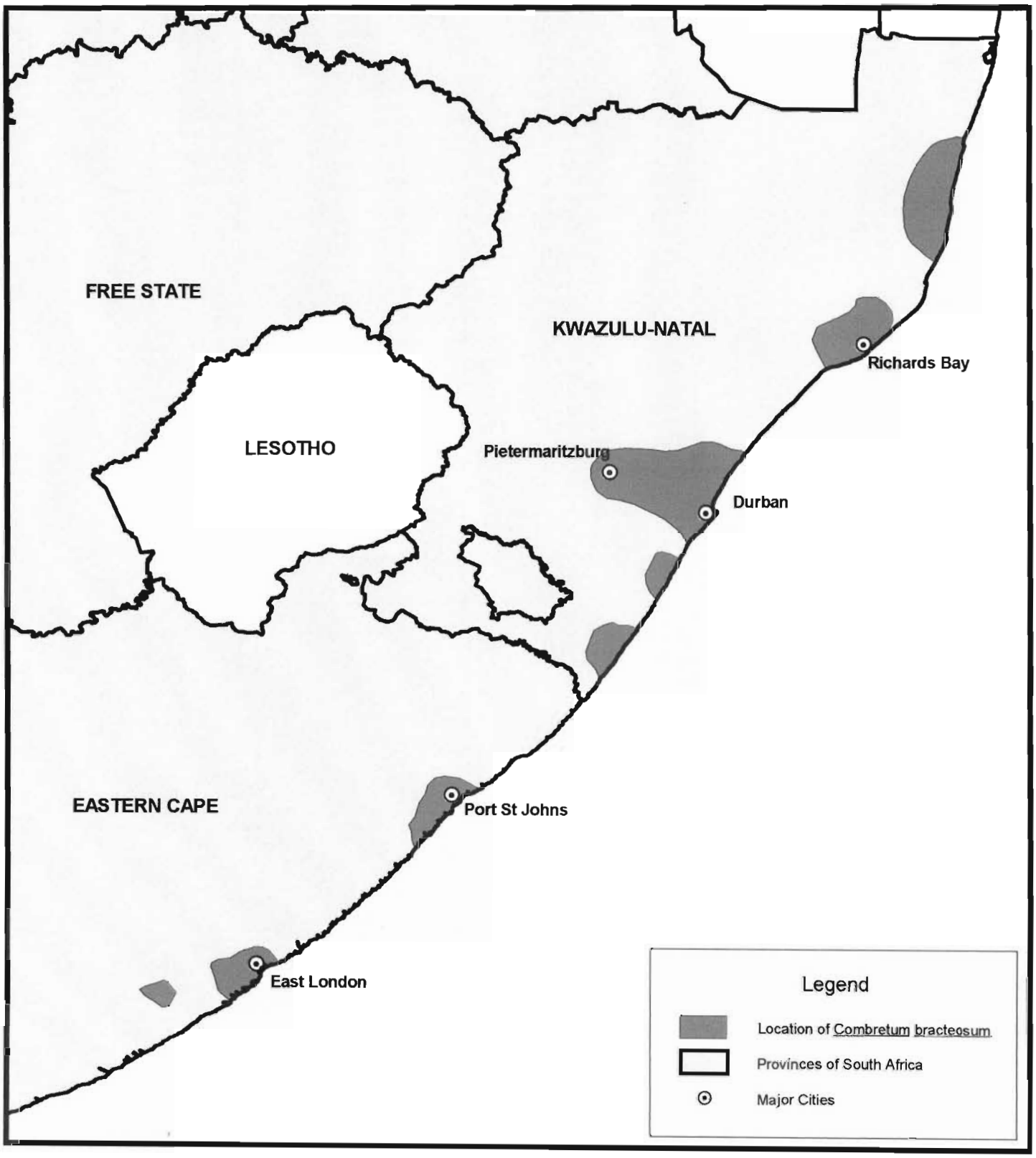


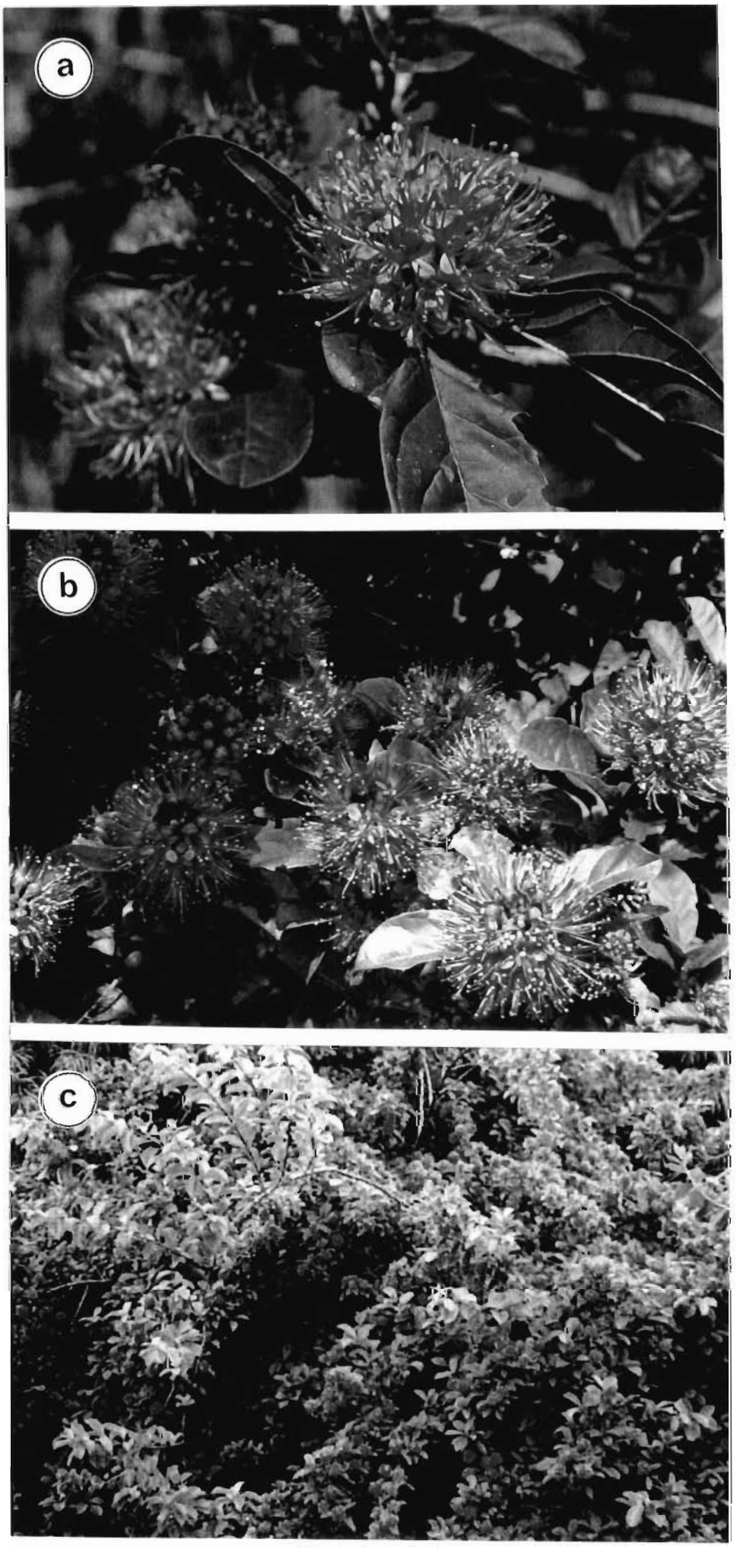
Figure 2: Distribution of *Combretum bracteosum* in South Africa

species, of which some thirty occur in South Africa (ESTERHUYSE, 1995). *Combretum* species bear 4-winged fruit with the exception of one species with 5-winged and one with wingless fruit. Other members of South African Combretaceae are the genera *Pteleopsis* (stink bushwillows), *Terminalia* (cluster-leaf trees), *Lumnitzera* (Tonga mangrove trees) and *Quisqualis* (quisqualis).

Combretum bracteosum is endemic to southern Africa, occurring only at low elevations in the coastal belt of Natal and the eastern Cape (Figure 2). It is found in dune forest, along stream banks, marginal to riparian fringe forest and in wooded kloofs. Where there is no supporting vegetation, this multi-stemmed, dense shrub, is 2-4 m high and spreads to a diameter of 4-5 m (Plate 1 c). However, it has scandent tendencies and has been observed to climb to as high as 8 m above ground in adjacent trees. Shrubs are topped by numerous, ascending, arcuate, usually branched stems, the terminals of which are trailing and sinuous as though seeking support. Where such stems make contact with available support they coil around this in a right-hand helix (CARR, 1988).

Flowering is profuse and spectacular (Plate 1 c) and usually takes place in September or October. On rare occasions it has been seen flowering as late as May. Inflorescences are in the form of terminal spikes on short (35-50 mm) leafy laterals (Plate 1 b), of which the latter are axillary, in pairs, or in whorls of three, usually along armed stems. Such spikes, have up to 24 flowers which are densely grouped, each having a basal leafy bracteole, which is petiolate. The petals are spathulate, up to 7 mm long and 3 mm wide, veined, bright red and splayed, their backs are minutely puberulous and their margins ciliate, the hairs being gold-coloured. Petals bleach white when the flower is fully open. The red filaments are exserted up to 11 mm (Plate 1 a). The anthers are plum-coloured and the tapering style is red with a green tip and projects out as far as the filaments (COATES-PALGRAVE, 1981; CARR, 1988).

The fruit of *Combretum bracteosum* differ from that of other southern African species in


a
b
c

being wingless. The fruit is a nut, almost spherical in shape (Plate 2 c). The nut has five sectors, between each of which is a shallow furrow running from the base round to the apex. The diameter is up to 23 mm and the stipe, which is about 2 mm in diameter, is about the same in length. The fruit, when ripe is coffee-coloured - occasionally with some red-brown or chocolate-tinged areas. It has a hard outer shell about 1 mm thick with a softer fibrous lining, 3 mm or more thick. The seed is also coffee-coloured but is darkened to a chocolate brown around the hilum. The general appearance of the seed is reminiscent of a small walnut. Occasionally there can be two seeds in a fruit and these have one side flattened (CARR, 1988). Although in some *Combretum* species the seed is toxic, the seed of *Combretum bracteosum* is said to be palatable. This may account for the paucity of the fruit available to horticulturists. The seeds, when ingested cause a prolonged hiccough - hence the common name "hiccough nut" or "hiccough creeper".

1.1 Use of Combretaceae in Traditional Medicine

Reports in the literature dealing with the role of Combretaceae in ethnobotany (WATT and BREYER-BRANDWIJK, 1962; KOKWARO, 1976; GELFAND *et al.*, 1985) indicate that traditional healers throughout Africa have confined themselves almost exclusively to the use of species from the genus *Combretum* and to a lesser extent the *Terminalia* in the treatment of a wide range of ailments (Table 1). Although the use of the leaves and bark from *Combretum* species is widespread, the fruit, which in most cases are produced in great abundance, are never used in medicine because of their reported toxicity to humans (ROGERS and VEROTTA, 1996).

Like many other Combretaceae, *Combretum bracteosum* is currently of no great economic value. It is occasionally planted for ornamental purposes, however, the horticultural potential of this beautiful red-flowered, spring-blooming species has never been optimized. The reason for this is apparently because propagation is difficult. Although no mention was made of *C. bracteosum* in Table 1, McGAW *et al.* (2001) in investigating the biological activity in *Combretum* species, showed that the leaves, when extracted using ethyl acetate,

Table 1: Some Medicinal Uses of the Combretaceae in Africa

Plant Name	Location	Ailment	Reference
COMBRETUM			
C. apiculatum Sond.	E. Africa S. Africa Zimbabwe	Snake bite, scorpion bite, bloody diarrhoea, leprosy Leaf decoctions for steam baths or administered for abdominal disorders, conjunctivitis Anthelmintic, anti-inflammatory and DNA damaging properties Leaves for weak body	HUTCHINGS *et al.*, 1996; ROGERS and VEROTTA, 1996; M^{c}GAW *et al.*, 2001
C. caffrum (Eckl. & Zeyh.) Kuntze	Africa	Root bark charm for harming enemy	WATT and BREYER-BRANDWIJK, 1962
C. decandrum	Africa	Bilious, burns, fever, hematuria, malaria	JOHNSON, 1999
C. erythrophyllum (Burch.) Sond.	S. Africa Zimbabwe Botswana	Small volumes of roots administered as a fattening tonic for dogs Abdominal pain (roots or leaves), venereal diseases (roots), coughs (leaves) Infertility and during pregnancy to facilitate labour Suspected of causing fatal human poisoning At least 14 different unidentified bacterial inhibitors Roots are a cure and prophylactic against venereal disease, and are administered vaginally as an aphrodisiac Reduce size of vaginal orifice Roots have purgative effect Root decoctions venereal disease; ointments applied to penile and vulval swellings	GELFAND *et al.*, 1985; HEDBERG and STAUGÅRD, 1989 VEALE *et al.*, 1992; HUTCHINGS *et al.*, 1996; ROGERS and VEROTTA, 1996; MARTINI and ELOFF, 1998; VAN WYK and GERICKE, 2000
C. fragrans F. Hoffm.	E. Africa Zimbabwe	Chest coughs, syphilis Aphrodisiac	ROGERS and VEROTTA, 1996
C. glutinosum	Senegal	Hepatic disease, antihypertensive, diuretic, bronchial disease	ROGERS and VEROTTA, 1996

Plant Name	Location	Ailment	Reference
C. hereroense Schinz	Botswana E. Africa Zimbabwe S. Africa Somalia	Venereal diseases and pains in the body (roots), ointments to penile and vulval swellings Bilharzia prophylactic Headache, infertility in women Abdominal pain and venereal diseases (roots administered as enema) Heart disease and heart-burn (bark) Anthelmintic and DNA damaging properties Young dried shoots for tonsilitis and coughs	PALMER and PITMAN, 1972; SAMUELSON *et al.*, 1992; HUTCHINGS *et al.*, 1996; ROGERS AND VEROTTA, 1996; M^{c}GAW *et al.*, 2001
C. imberbe Wawra	S. Africa Zimbabwe Namibia	Coughs and colds Anti-inflammatory and anti-schistomsomal properties Diarrhoea, to drive away bad spirits, bilharzias Cultural and religious significance	ROGERS and VEROTTA, 1996; JOHNSON, 1999; VAN WYK and GERICKE, 2000; M^{c}GAW *et al.*, 2001
C. krausii Hochst.	S. Africa	Anti-diarrhoeals, lotions for eye infections and anti-septic for wounds Tonic to stimulate appetite (root), enema to cleanse urinary system and strengthen spinal column Cleanse wounds	HUTCHINGS *et al.*, 1996; VAN WYK *et al.*, 1997.
C. microphyllum Klotzsch	Zambia Zimbabwe French Sudan Nigeria Senegal	Lunacy Lucky charm; sores and abscesses Biliary fever, cholic and vomiting	OLIVER-BEVER, 1986; ROGERS and VEROTTA, 1996.

Plant Name	Location	Ailment	Reference
C. molle R. Br. Ex G. Don	E. Africa	Hookworm, stomach ache (inner bark infusions), snake bite antidote (leaves and root together), leprosy, leaf decoctions used to wash febrile patients, dysentery, chest complaints, anthelmintic (root infusions)	IRVINE, 1961; HEDBERG *et al.*, 1982; GELFAND *et al.*, 1985; HUTCHINGS *et al.*, 1996; VAN WYK and GERICKE, 2000; M^{c}GAW *et al.*, 2001
	Zambia	Boiled leaves used as hot compresses and steam inhaled for headaches	
	Malawi	Anthelminthic, snake bite antidote (leaves and root together)	
	Zimbabwe	Abdominal pains (inner bark infusions), diarrhoea (root), headaches, convulsions, infertility in women (roots), to stop bleeding after childbirth (roots), to fatten babies, as a dressing for wounds, aphrodisiac, backache, difficulty walking believed to be caused by sorcery (roots). Roots used for infertility and treating post-partum bleeding, wound dressing (leaves), abortion (roots), constipation and swollen abdomen	
		Anti-inflammatory and anti-schistosomal properties	
	S. Africa	Wound dressing, abortifacients and anthelmintics, stomach ache, colic and fevers	
		Worms (bark), infertility, intestinal worms, laxative	
	Ghana	Wounds, snakebite antidote, stomach complaints (leaves and roots)	
C. mossambicense (Klotzsch) Engl.	S. Africa	Anti-inflammatory, anthelmintic and DNA damaging properties	M^{c}GAW *et al.*, 2001
C. mucronatum	Nigeria, Sierra Leone	Preventative of Guinea worms	JOHNSON, 1999
C. paniculatum Vent.		Expel retained placenta	VAN WYK and GERICKE, 2000
C. platypetalum Welw. ex Laws.	Zambia	Swelling caused by mumps	ROGERS and VEROTTA, 1996
	Zimbabwe	Pneumonia, abdominal pains, diarrhoea, antiemetic, dysmenorrhoea, infertility in women, earache, epistaxis, haemoptysis	
	S. Africa	Stomach pain and severe vomiting	

Plant Name	Location	Ailment	Reference
C. racemosum	Ghana	Internal parasites, vermifuge	OLIVER-BEVER, 1996; JOHNSON, 1999
C. zeyheri Sond.	E. Africa Tanzania Zambia S. Africa Zimbabwe	Toothache, cough Scorpion bite, diarrhoea with blood To arrest menstrual flow (powdered bark), eye lotion (bark ashes), embrocation (crushed leaves mixed with oil, diarrhoea Diarrhoea with blood, abdominal disorders, eye infection, embrocation to ease backache, haemorrhoids. Gall stones (bark) Bloody diarrhoea (root infusion)	WATT and BREYER-BRANDWIJK, 1962; GELFAND *et al.*, 1985; BREYTENBACH and MALAN, 1989; POOLEY, 1993; HUTCHINGS *et al.*, 1996; ROGERS and VEROTTA, 1996
TERMINALIA			
T. brachystemma Welw. ex Hiern	Zimbabwe	Haematuria, bilious vomiting, constipation, diarrhoea	ROGERS and VEROTTA, 1996
T. brevipes	Somalia	Hepatitis, malaria	ROGERS and VEROTTA, 1996
T. brownii	Ethiopia	Hepatitis	JOHNSON, 1999
T. catappa	Dominican Republic	Bites, cough, diarrhoea, dysentery, fever, gastritis, insomnia, leprosy, mouth infection, nerves, piles, rheumatism, sore throat, stomach ache, thrush, wounds	JOHNSON, 1999
T. chebula	Egypt	Asthma, bronchitis, burns, conjunctivitis, cough, diarrhoea, inflammation, measles, tumours	JOHNSON, 1999
T. glaucescens	Sierra Leone Burkina Faso	Purgative, cough, diarrhoea, dysentery, jaundice, leprosy, sores, syphilis, toothache, wounds	JOHNSON, 1999

Plant Name	Location	Ailment	Reference
T. ivorensis	Burkina Faso Ghana	Rheumatism, sores, wounds	JOHNSON, 1999
T. macroptera	Sudan	Boils, sores, wounds	JOHNSON, 1999
T. phanerophlebia Engl. & Diels		Diabetes (powdered bark)	HUTCHINGS *et al.*, 1996
T. sericea Burch. ex DC.	E. Africa	Bilharziasis (powdered roots), stomach troubles	COATES-PALGRAVE, 1981; GELFAND *et al.*, 1985; HEDBERG and STAUGÅRD, 1989; HUTCHINGS *et al.*, 1996; ROGERS and VEROTTA, 1996; VAN WYK, 1997; JOHNSON, 1999
	Botswana	Various gastric and gynacological disorders, venereal diseases, general weakness, sore throats, nosebleeds, warts	
	S. Africa	Stomach disorders (roots), bilharziasis (powdered roots), diabetes (bark), decoctions and infusions to treat pneumonia (hot infusions of outer root layers), lotions for eye infections (root), wounds (leaves or bark), infertility and venereal diseases	
		Roots emetic and/or inoculating powder, rubbed into scarifications for ailments caused by witchcraft (takes the form of pain in the chest) - sometimes these symptoms refer to TB or rheumatism	
	Tanzania	Stomach disorders, bilharziasis (powdered roots)	
	Zimbabwe	Diarrhoea (root), colic, epistaxis, prolapsed rectum of infants, backache, to widen vagina, wounds, abdominal pains, worms on arms, antiemetic, infertility in women, tonic, depressed fontanelle, sore throat, gonorrhoea, bilharziasis, abortion, dilated birth canal.	
T. stenostachya Engl. & Diels	Zimbabwe	Epilepsy, antidote for poison	ROGERS and VEROTTA, 1996

displayed a 100% inhibition of anti-inflammatory activity. A decoction made from roots is taken orally to treat diarrhoea and an infusion made from root bark is used to treat bilharziasis. Green leaves placed on hot coals produce smoke which is inhaled to relieve coughs and colds. Coughs are sometimes also treated with an infusion made from flowers (VENTER and VENTER, 1996).

1.2 Propagation and Cultivation

In most tree breeding practices, sexual or vegetative methods for propagation are used. Sexual reproduction is usually called the natural way of propagation, while propagation by vegetative means is considered to be artificial. A number of species reproduce both sexually and vegetatively, although sexual reproduction seems to be the most important. Sexual reproduction is essential for producing genetic variation, which is balanced by selection (natural and artificial) to keep the population adaptable. Vegetative reproduction is used to maintain the genotype best adapted to the site where the tree is growing or is used for multiplication of desirable trees.

In addition to the natural ways of vegetative propagation, breeders have developed many different methods for vegetative propagation, not all of which can be applied to every tree species. The reasons for this are the differences between species, the genetics and differentiation process of the tree, the developmental and physiological stages, which are all endogenous, and a number of exogenous factors. The exogenous factors can easily be controlled, if their importance and influence on propagation are known.

Limited attempts have been made to propagate various *Combretum* species (Table 2). In order to promote the horticultural potential of *Combretum bracteosum* the development of easy and rapid propagation methods have to be undertaken. Traditional vegetative propagation methods, such as cuttings, as well as the more modern technique of tissue culture were considered important methods to test as these were likely to be the most convenient, and most accessible methods to horticulturists.

Table 2: Combretaceae plants successfully propagated by means of cuttings (CARR, 1988)

Source Material	Notes
Combretum caffrum	Amenable from spring cuttings
Combretum kraussii	Sometimes gave reasonably good results from spring cuttings
Combretum moggii	Work not advanced enough for the degree of success to be estimated
Pteleopsis myrtifolia	Spring cuttings using previous years watershoot material have been partially successful
Quisqualis parviflora	
Terminalia	Cuttings have been unsuccessful

Further investigations on germination patterns of *Combretum bracteosum* seeds were also deemed necessary, as overcoming their poor rate of germination would improve their popularity as a garden plant. The poor viability of the seed has led propagators to seek alternative methods of multiplying the species, as relying on seed germination alone is too risky. Literature suggests that the wingless pericarp plays an influential role in the poor proportion of seeds able to germinate (CARR, 1988). By determining whether it is the pericarp alone that reduces the success rate of *Combretum bracteosum* seed germination, further ideas to overcome this restriction can be developed in order to optimize propagation for the commercial market.

In its natural habit, *Combretum bracteosum* fits well in a Botanical Garden or natural bush. Numerous authors state that *C. bracteosum* has great horticultural potential, but its large volume and scandent tendencies make it impractical to the smaller property owner. Supported by the establishment of improved methods of propagation, successful dwarfing could boost this plants' popularity in the commercial market even further as, by reducing the size of the plant, it would be far more appealing to both horticulturists and urban gardeners.

Chapter 2
Seed Germination of *Combretum bracteosum*

2.1 Introduction

Viability of seeds of many indigenous trees varies considerably from year to year. It is thought that for those species affected, unfavourable conditions at the time of flowering may be the cause. Unseasonable cold, heavy rains and non-availability of the necessary pollinators could have an adverse effect on pollination and so, on the production of fertile seed. With *Combretum* species, excessively dry conditions during and after flowering reduce the amount of fruit produced drastically and inhibit development of those fruits that persist on the tree (CARR, 1988; DALLING and VAN STADEN, 1999).

Only one report was found where reference was made to the germination of *Combretum* species. CARR (1988) reported that a number of species germinated poorly under the former Transvaal highveld conditions (altitude 1600 m, severe winters, maximum temperatures frequently exceeding 30°C).

Wherever possible, CARR (1988) investigated the preparation of different *Combretum* species' seeds for sowing. In most instances, seed was sown as soon as it became available, so the question of how long seed may be stored without loss of viability has not been investigated for most *Combretum* species. Table 3 however, indicates the length of time other *Combretum* species seeds were stored, with no negative effect on subsequent germination. Three of these species are from semi-desert areas, where rainless periods may endure for several years and these plants may have become genetically adapted to overcome long dormancy periods (CARR, 1988).

Removing the seed from the fruit before sowing was recommended by CARR (1988), provided this could be accomplished without the embryo being damaged. This procedure not

only allows seed to be inspected, but it also allows germination to proceed without any physical restriction other than that imposed by the testa. If seed could be excised undamaged from the woody pericarp, the proportion of seeds germinating would probably increase. It is also thought that germination time would be reduced from the usual 20-60 days to a matter between five and eight days (CARR, 1988).

Table 3: Storage times determined for seeds of various Combretaceae species (CARR, 1988)

Species	Time period (years)
C. apiculatum subsp. *leutweinii*	7½
C. oxystachyum	17
C. psidioides subsp. *dinteri*	11
C. psidioides subsp. *psidioides*	4
M. tetrandrus subsp. *australis*	7
T. randii	7
T. stuhlmannii	6

Seed production of *Combretum bracteosum* is not always prolific and their germination has not been thoroughly investigated. It was for this reason that certain aspects of germination were investigated.

Recalcitrant seed germination is initiated shortly after the seeds are shed from the parent plant. The sub-cellular events associated with germination can then proceed in storage (even under conditions of slight water loss), up to the initiation of cell division and extensive vacuolation. As germination proceeds beyond the initiation of cell division, the seeds become increasingly sensitive to desiccation and the amount of water loss tolerated declines until ultimately, water becomes limiting and viability is lost. Because of this increasing sensitivity to desiccation, seeds dried rapidly immediately after shedding can tolerate a greater degree of water loss than those which are dried slowly. Increasing desiccation

sensitivity has been related to the requirement for an increasing degree of cytomatrical organisation with the onset of full metabolism upon germination (FARRANT *et al.*, 1988). Dehydration results in irreversible metabolic disruption.

Seeds may be broadly divided into two categories, based on whether or not they undergo a period of maturation drying as the final pre-shedding developmental phase. In 1973, ROBERTS introduced the terms 'orthodox' and 'recalcitrant' to describe the storage behaviour of seeds. The majority of seed species are 'orthodox', they terminate their development by maturation drying, during which there is a metabolic switch from the developmental mode to that of germination (KERMODE and BEWLEY, 1988). After they are shed, the low moisture content of such seeds remains in equilibrium with the relative humidity of the atmosphere. Orthodox seeds can generally be further dried to moisture contents in the range of 1-5% without damage. In this dehydrated state the seed can resist the vicissitudes of the environment, and unless dormant, will resume full metabolic activity, growth and development when conditions conducive to germination are provided. Because of these properties, these seeds can be stored for long periods (ELLIS and ROBERTS, 1980).

Recalcitrant seeds, which are almost invariably large, do not undergo maturation drying, and are shed at relatively high moisture contents (*Combretum bracteosum* - moisture content 51%). Such seeds are highly susceptible to desiccation injury, and thus are not storable under conditions suitable for orthodox seeds. Furthermore, most recalcitrant seeds are sensitive to chilling injury at lowered temperatures (FARRANT *et al.*, 1988). Storage at ambient temperatures, in fully imbibed states usually results in microbial contamination, and even if anti-microbial agents are applied, the period of viability appears to remain short, varying from a few weeks to months, depending on the species (FARRANT *et al.*, 1988). Currently cryopreservation is under investigation as a suitable long-term storage method for recalcitrant seeds (BERJAK *et al.*, 2000; CHANDEL *et al.*, 1995; FU *et al.*, 1990; PAMMENTER and BERJAK, 1999; WESLEY-SMITH *et al.*, 1999).

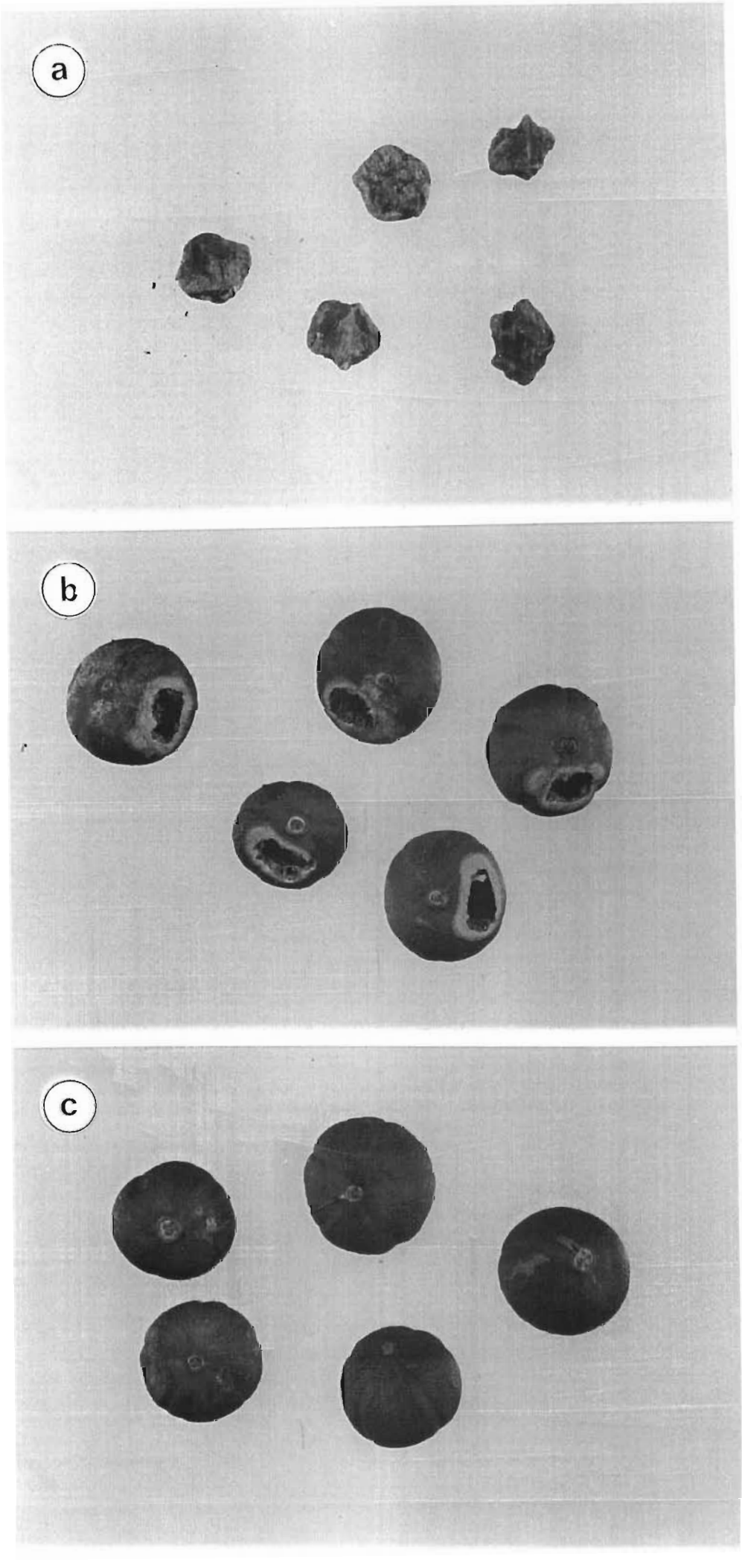
This investigation aimed to determine the effects of dormancy on germination as well as conditions most suitable for optimal seed gemination of *Combretum bracteosum*. Factors such as chilling, leaching, light and temperature were considered as they are most applicable to the natural environmental conditions at low elevations of the KwaZulu-Natal and eastern Cape coastal belt - the natural habitat of this shrub (Figure 2).

2.2 Materials and Methods

Combretum bracteosum (Horchst.) Brandis. seeds were obtained from Kirstenbosch National Botanical Gardens (33°59′00″S 18°26′00″E). These mature seeds from the immediate past fruiting season (December 1997 - March 1998), were collected directly off the trees.

All germination studies were conducted in plastic containers (diameter 70 mm; depth 30 mm) on two layers of moist filter paper. Dishes were covered with a lid and then placed in the respective incubation conditions. Each replicate comprised five seeds, with five replicates per treatment. Germination was recorded every second day and was considered complete once the radicle was protruding about 2 mm through the covering structures or from the excised embryo where appropriate.

Besides the small quantity of seeds that these *Combretum bracteosum* shrubs generally produce, it has been suggested that the hard pericarp surrounding the seeds provides a physical barrier to germination, adding to the low number of seedlings produced by these shrubs annually. Germination experiments were carried out using excised embryos (Plate 2 a), partly scarified (Plate 2 b) and non-scarified seeds (Plate 2 c), in order to determine the extent to which the hard pericarp influences germination. As an alternative or a co-factor to the pericarp being a physical barrier, an environmental factor such as temperature could have a significant influence on the rate and mode of germination. To determine these effects on germination, incubation temperatures of 20, 25, 30 and 35°C in the light, or at 25°C in complete darkness, were tested (germination was recorded under green "safe light" 0.5 $\mu mol.m^{-2}.s^{-1}$).


a
b
c

Seed fresh mass was recorded daily in order to follow water imbibition. Seeds with an intact pericarp and excised embryos, were incubated at ambient temperatures and light conditions.

Excised embryos were also imbibed on filter paper moistened with an aqueous leachate obtained from the removed pericarps, to test for the possible presence of inhibitors. Intact seeds were stratified by incubating them in a moist plastic bag, in complete darkness at 4°C, for one and two months respectively. Following stratification, seeds were incubated in plastic containers at 25 and 30°C respectively (DALLING and VAN STADEN, 1999).

2.3 Results and Discussion

Excised embryos imbibed water rapidly under ambient conditions (Figure 3). Within hours of placing the wrinkled embryos onto moist filter paper they became fully swollen and commenced germination after eight days, reaching 100% germination after day ten. Water uptake of intact seeds was slower but the results presented in Figure 4 indicate that the pericarp does not restrict imbibition. The presence of the pericarp did however, reduce the rate of germination. Only after 49 days was 100% germination achieved. The reduced germination rate cannot be caused by the pericarp restricting the uptake of water, as the mass increase of the seed is steady from day 1. This leaves the possibility that the pericarp acts as a physical barrier to the emerging radicle, or produces an inhibitory substance resulting in delayed germination.

Temperature did have an effect on the germination of both excised embryos (Figure 5 a), partly scarified (Figure 5 b) and intact seeds (Figure 5 c). Excised embryos germinated rapidly at 20°C, 25°C and 30°C. A linear rate of germination was recorded at 25°C. Thirty-five degrees centigrade was not suitable for excised embryo germination (Figure 5 a). Incubation of the partly scarified *Combretum bracteosum* seeds at thirty and thirty-five degrees centigrade resulted in a poor rate of germination (Figure 5 b). The lower temperatures (20°C and 25°C) induced the best germination. Although 25°C only induced

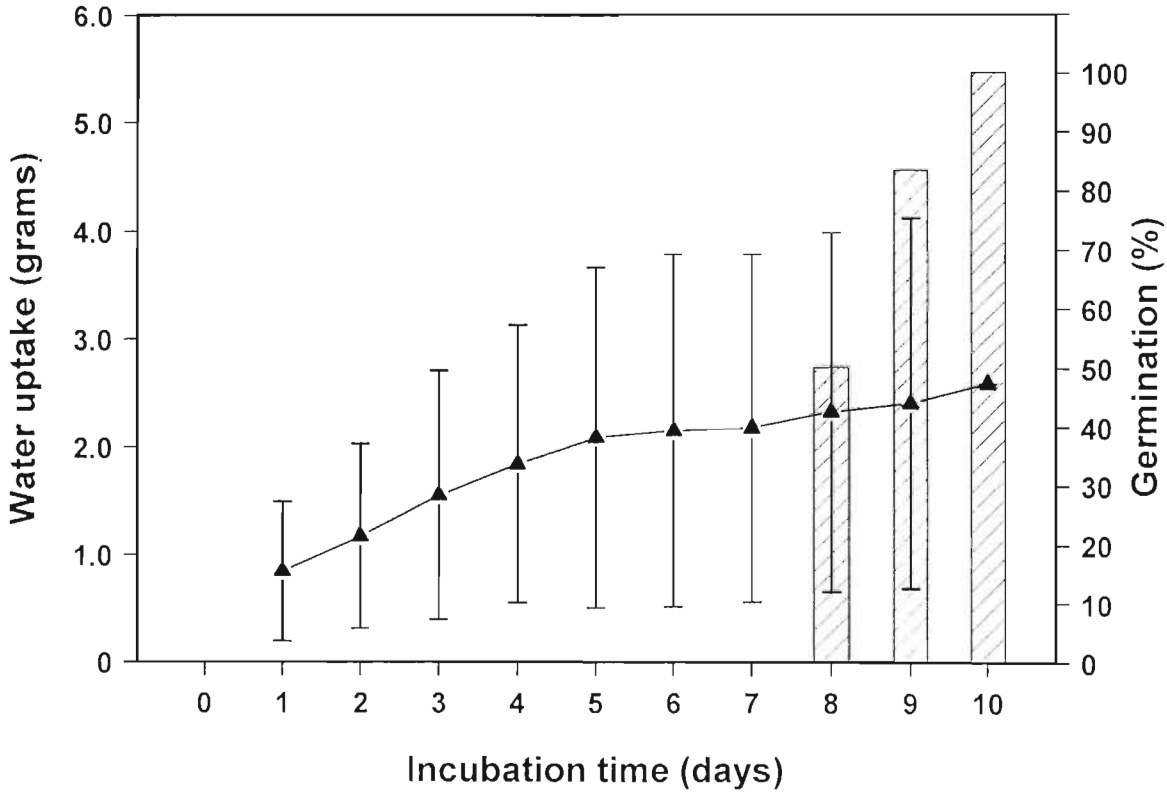


Figure 3: Imbibition and germination rates of excised *Combretum bracteosum* embryos. Bars indicate S.E.

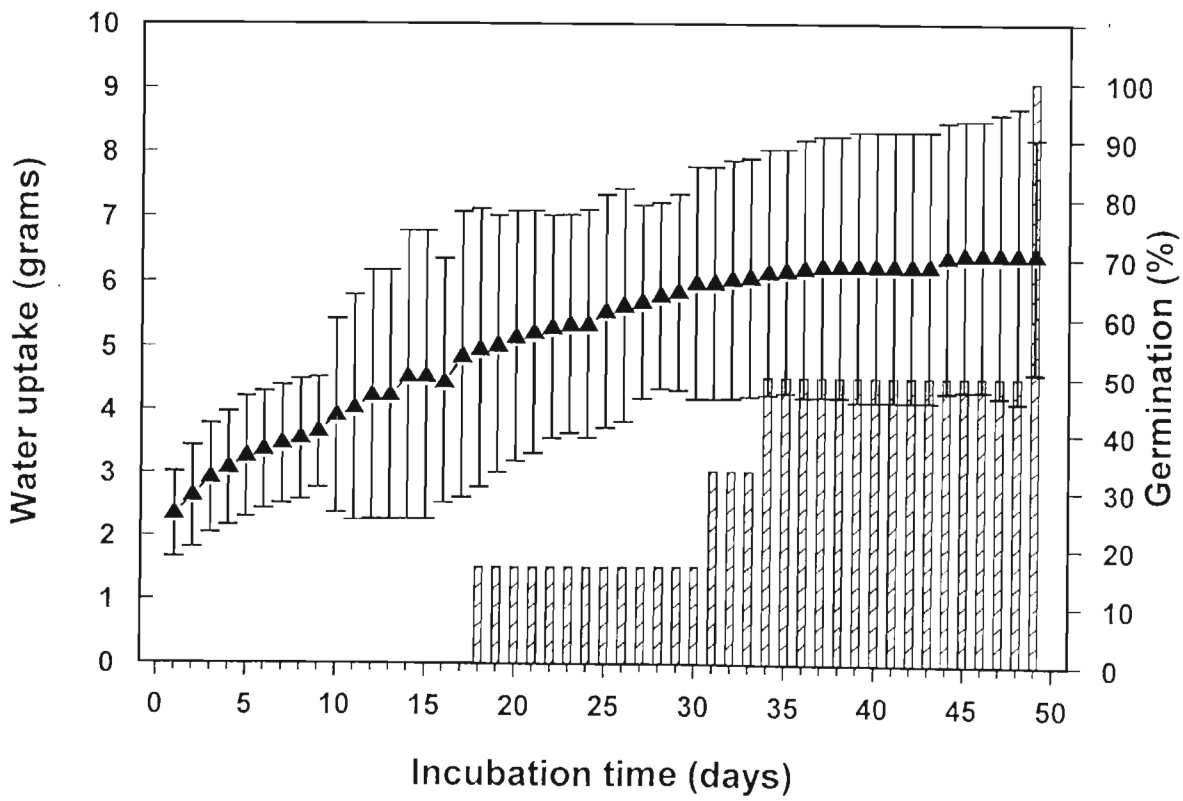
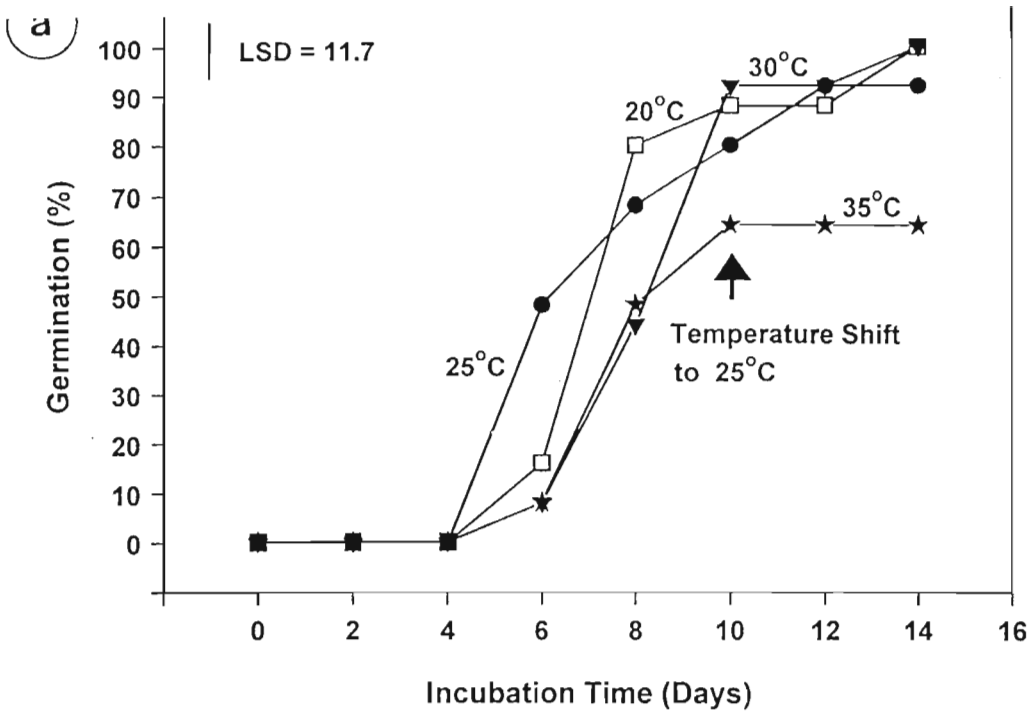
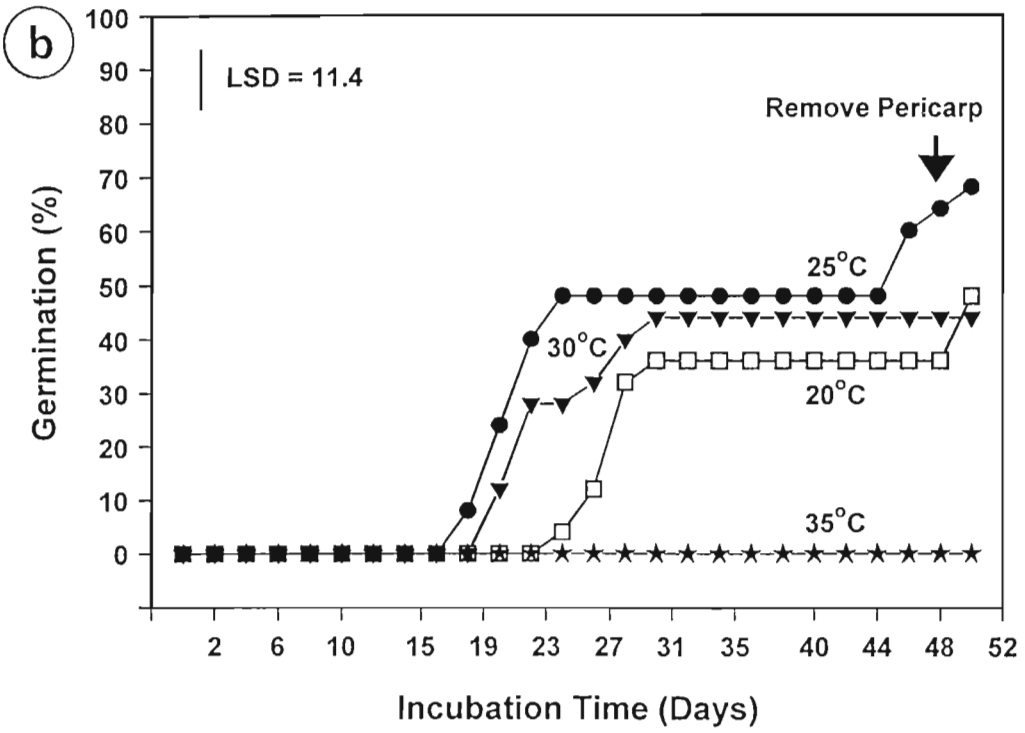
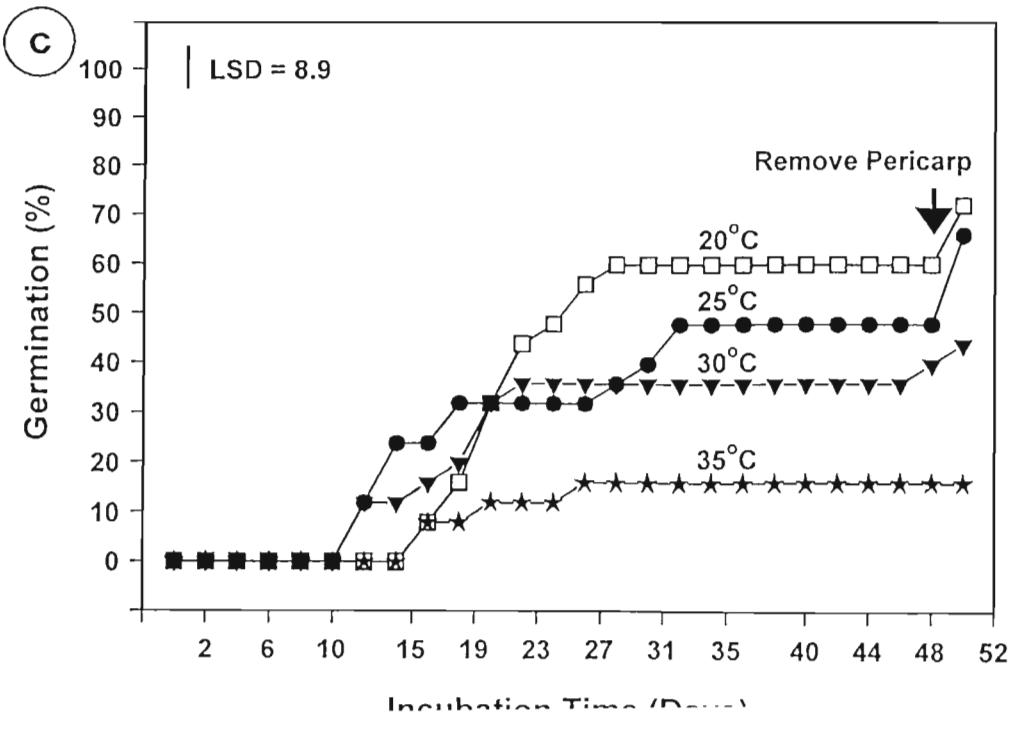


Figure 4: Imbibition and germination rates of intact *Combretum bracteosum* seeds Bars indicate S.E.


a
LSD = 11.7
30°C
20°C
35°C
25°C
Temperature Shift
to 25°C
Germination (%)
Incubation Time (Days)


b
LSD = 11.4
Remove Pericarp
25°C
30°C
20°C
35°C
Germination (%)
Incubation Time (Days)


c
LSD = 8.9
Remove Pericarp
20°C
25°C
30°C
35°C
Germination (%)

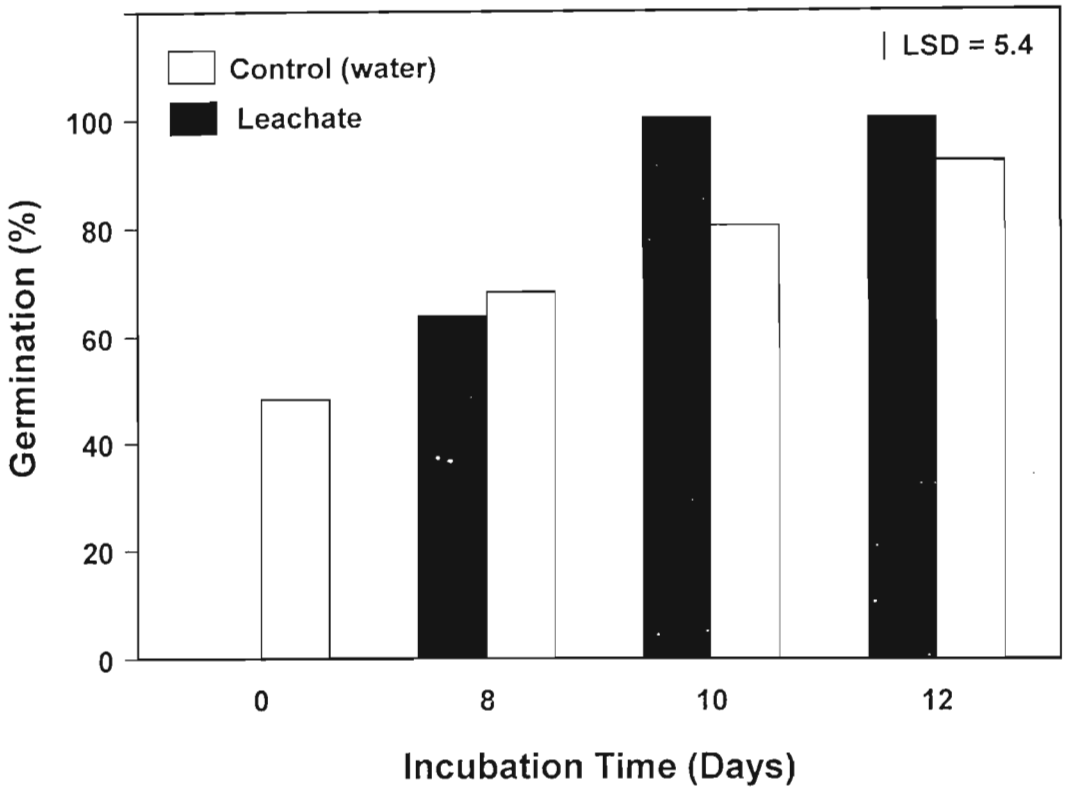


Figure 6: The effect of pericarp leachate on the germination of excised *Combretum bracteosum* embryos

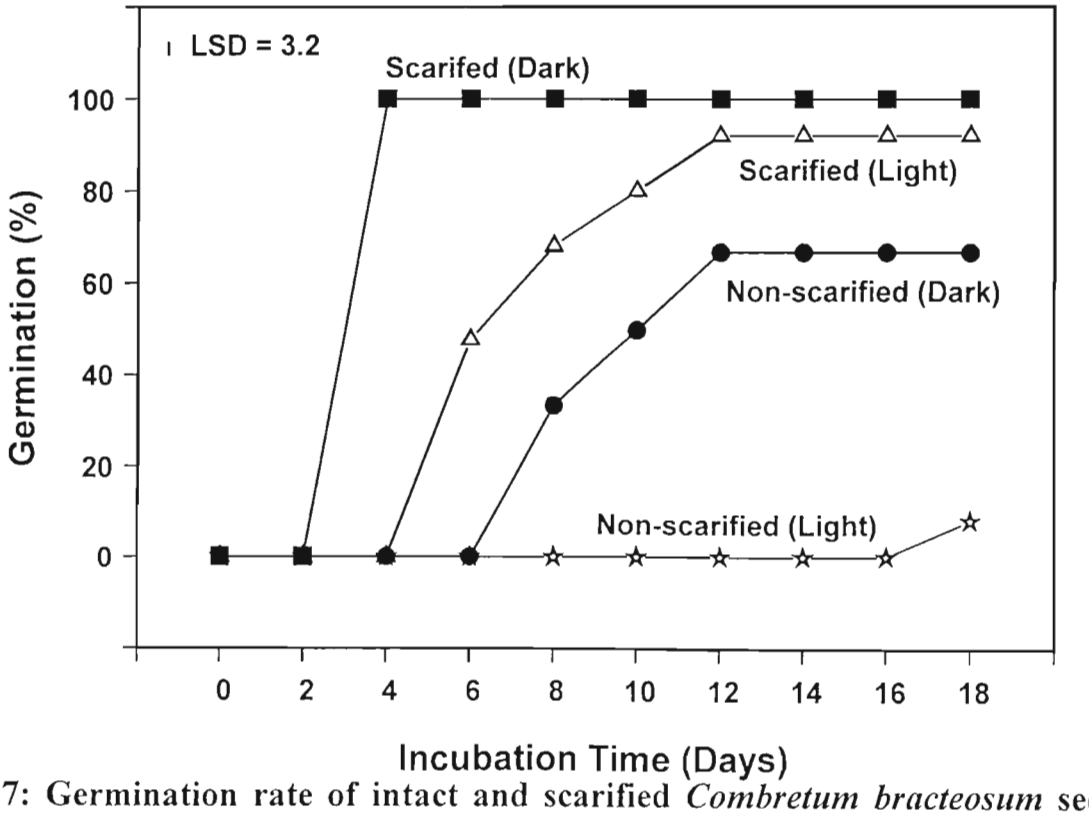


Figure 7: Germination rate of intact and scarified *Combretum bracteosum* seeds incubated at 25°C in the dark and light

about 47% of the seeds to germinate, a further 17% of the remaining ungerminated embryos were still able to germinate after the complete removal of the pericarp at 47 days of incubation. The remaining embryos were unable to germinate as they had already rotted.

That the presence of the pericarp or part thereof does affect germination is further emphasized by the results presented in Figure 5 c. With an intact pericarp, germination was much lower, reaching about 50% after 25 days. The detrimental effect of 35°C was most pronounced with the intact seeds, which all rotted. The lowest temperature (20°C) was also less efficient with non-scarified seeds, with less than 40% germinating in 48 days. Increasing the incubation temperature above 20°C prompted microbial attack to which embryos and seeds were highly susceptible. Incubation of seeds at 25°C appeared to be able to overcome microbial invasion, with almost 70% of seed germinating over the experimental period. The presence of the pericarp did affect radicle protrusion and had some mechanical influence. Inspection of non-germinated intact seeds after 48 days incubation revealed that the radicles had in fact commenced elongating and had produced some lateral roots which were unable to protrude from the covering structures. The tips of the radicle and roots were black, wilted and had begun rotting.

Compounds leached from the pericarp did not play an important role in the germination of the excised *C. bracteosum* embryos (Figure 6), suggesting that none of them are inhibitory. Excised embryos germinated significantly (statistically) faster in the dark than in the light (Figure 7). Germination of intact seeds followed the same trend.

As the tissue of *C. bracteosum* seeds remain hydrated at maturity (51% moisture content), and were intolerant of stratification, they appear characteristically recalcitrant. Incubating the seeds in moist conditions with low temperatures (4°C) for both one and two months had detrimental effects on the viability of the seeds. Subsequent incubation at 25 and 30°C resulted in a response from the one-month-stratified seed only, with an eight and four percent germination respectively. None of the seeds stored for two months germinated. As

recalcitrant seeds do not undergo a period of dessication at maturity, they maintain their high metabolic activity (DODD, 1981). Being unable to photosynthesize, their food reserves are wasted and thus their viability is lost rapidly.

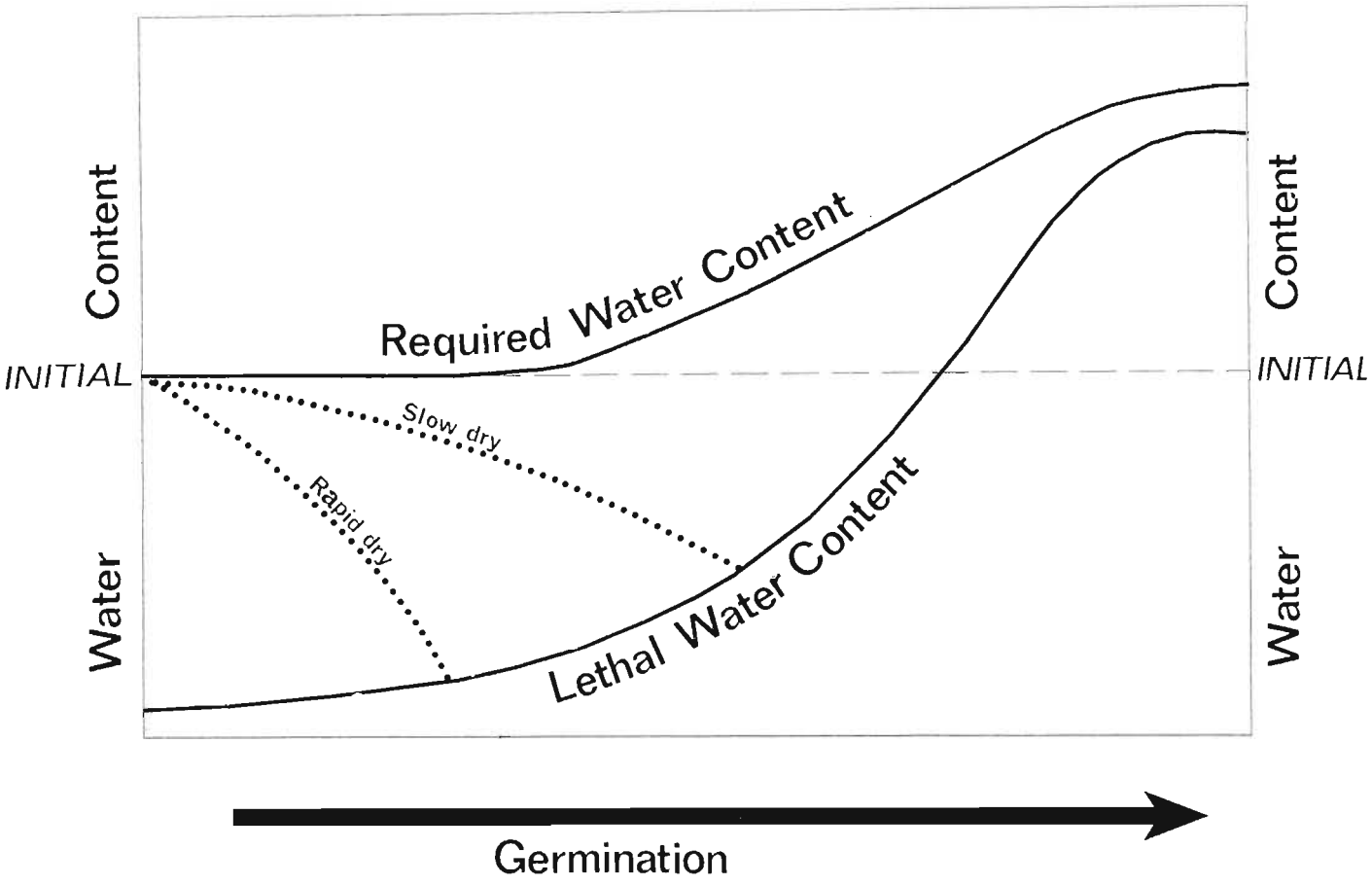


Figure 8: A model proposed to explain the storage and germination behaviour of desiccation-sensitive recalcitrant seeds. Abscissa: developmental sequence in the embryonic axis during germination; Ordinate: axis water content (Modified after FARRANT *et al.*, 1986 a)

In 1984, BERJAK and co-workers suggested that the desiccation sensitivity of recalcitrant seeds, with their water content maintained at the same level as when newly-shed, may be due to the initiation of germination-associated events in storage (FARRANT *et al.*, 1986 b; FARRANT *et al.*, 1988). Such seeds may then be comparable to imbibed, germinating,

orthodox seeds, which have become sensitive to desiccation.

Figure 8 depicts a model proposed to describe the behaviour of recalcitrant seeds in general, but which is based on the behaviour of the seeds of *Avicennia marina* (FARRANT *et al.*, 1986 a). The abscissa represents the germination pathway and although time-dependant, it is not necessarily linearly so, and the actual shapes of the curves and the absolute values of the water contents will vary amongst species.

When newly shed, the seeds with a high moisture content are metabolically active, but probably appear relatively quiescent (FARRANT *et al.*, 1985; FARRANT *et al.*, 1988). A short while after shedding, sub-cellular changes are initiated which are similar to those occurring during early germination, these changes continue in the short-term (even in the absence of additional water), until a critical proportion of bulk water is lost (BERJAK *et al.*, 1984; PAMMENTER *et al.*, 1984; FARRANT *et al.*, 1986 b; FARRANT *et al.*, 1988). This probably constitutes a protection mechanism functioning in the natural environment, where some water loss is likely to be inevitable. It may well be that the embryo, or embryonic axis is able to absorb water for some time from the endosperm under such conditions.

As more and more pathways are initiated as the germination-related events proceed (BERJAK *et al.*, 1984), the seeds become increasingly sensitive to desiccation and the amount of water loss tolerated declines (or the minimum lethal water content is raised). It is hypothesized by FARRANT *et al.* (1988) that an increasing proportion of the bulk water present would have structure imposed upon it by the newly-synthesised sub-cellular components, including the macromolecules themselves. The cyto-matrix would therefore become more complex and ordered, the order itself facilitating optimal cyto-matrical functioning. Loss of bulk water is suggested to have little effect on metabolism, other than perhaps a decline in rate of reactions. However, it is proposed that loss of structured water would result in disruption of certain metabolic pathways and the disorganisation of metabolism. As germination proceeds, less and less water can be lost without the initiation

of some metabolic disruption. With increasing loss of structured water there would be increasing disruption of metabolism accompanied by sub-cellular disorganisation, until ultimately a loss of stability of sub-cellular structures, including membranes, would result. Loss of stability in the latter would lead to loss of integrity of the plasmalemma, the tonoplast and other membranes, and so to the loss of viability.

Because the seeds become increasingly sensitive to desiccation, the rate at which they are dried can affect their viability characteristics (FARRANT *et al.*, 1985). If rapidly dried before they have proceeded to any extent along the germination pathway, they can tolerate a greater amount of water loss and thus survive at lower moisture contents. Seeds that are slowly dried, however, attain a more advanced stage of germination (the water level remaining sufficiently high to allow this during protracted drying) (FARRANT, *et al.*, 1988). Because of this, death occurs at higher moisture contents (Figure 8). *Combretum bracteosum* seeds, being relatively large in size, undergoes slow drying and so is unsuited to long term storage, as viability is lost.

It seems that there is a difference in the tolerance of different recalcitrant species to water loss. In this regard, there appears to be a continuum of recalcitrant seed-types, varying from less recalcitrant to highly recalcitrant (Table 4). Where they fall on the scale is suggested to relate in part, to the habitat to which they are adapted. It is thought that *Combretum bracteosum* seeds are classified as minimally-to-moderately recalcitrant.

2.4 Conclusion

Similar to the findings of CARR (1988) in respect of the germination of other *Combretum* species, the hard pericarp surrounding the *Combretum bracteosum* embryo acts as a mechanical barrier to the emerging radicle and roots. The hard pericarp did not impose any other dormancy inducing mechanisms in that the uptake of water was not restricted and the leachate from the pericarp was not inhibitory to the germinating seed. It was however, the inability of the emerging radicle to penetrate the hard pericarp rather than the viability of the

seed, that resulted in *Combretum bracteosum* seed germinating poorly. The recalcitrant characteristics of the seed, together with the influence exerted by temperature, and the mechanical hindrance of the pericarp, are probably the factors responsible for the inadequate number of seeds germinating under natural conditions.

Table 4: The continuum proposed to account for the varying degrees of recalcitrance (FARRANT *et al.*, 1988)

RECALCITRANT SEED TYPES		
MINIMALLY	MODERATELY	HIGHLY
- Fair amount of water loss tolerated - Lack of additional water - slow germination - Lower temperatures tolerated - Temperate/sub-tropical distribution. Examples: *Quercus* species, *Araucaria hunsteinii* and *Podocarpus henkelii*	- Moderate amount of water loss tolerated - Moderate germination rate in absence of additional water. - Most species are temperature-sensitive - Tropical distribution. Examples: *Theobroma cacao* and *Hevea brasiliensis*.	- Little water loss tolerated - Germination rapid in absence of additional water - Most species are temperature sensitive - Distribution tropical forests/wetlands. Examples: *Syzygium* species and *Avicennia marina*
← PREDICTED LONGEVITY IN STORAGE		

Chapter 3
Vegetative Propagation of *Combretum bracteosum* using cuttings

3.1 Introduction

The most important class of propagule utilized in the ornamental plant industry is cuttings (DAVIES *et al.*, 1994). In order to regenerate a new plant, cuttings must initiate an adventitious root system, after which the rooted cuttings can regenerate a new shoot system.

Vegetative plant propagation is used when there is an inability to produce viable seed, for plants that vary considerably in their genetic makeup, to perpetuate a particular form of plant (often juvenile), to increase the rate of propagation, to develop immunity to pests or an adaption to the habitat or to modify the growth habit (often dwarfing) of the plant (MAHLSTEDE and HABER, 1957). Propagation by means of cuttings has been a common practice for centuries and presently is second to seed as the most popular method of plant propagation (WRIGHT, 1973; BARLOW, 1994). Nevertheless, cuttings are not yet sufficiently successful to meet public demand in commercial nurseries (HAISSIG and DAVIS, 1994). The ease with which different varieties of plants can be multiplied by cutting methods varies widely, resulting in more elaborate methods often being resorted to with difficult subjects. Propagation by cuttings involves the removal of a vegetative portion of the stem, root or leaf of the parent plant and rooting it by subsequent manipulation. Of these, the method propagation from stem cuttings has by far the widest application (PEARSE, 1939). New plants produced are identical to the parent plant.

Current experience gained in propagating *Combretum* species by means of cuttings was obtained on the Transvaal highveld at an altitude of 1600 m. Winters are often severe, spring and early summer weather unpredictable and summer maximum temperatures frequently exceeding 30°C (CARR, 1988). These climatic conditions are not well suited

to that of *Combretum bracteosum,* leading to the belief that variation from the reported propagation results and growth rates could be expected in the plants' natural surroundings.

Treatment of cuttings with various substances to promote adventitious rooting is a well established concept, undoubtedly dating to the earliest endeavours to vegetatively propagate plants. Some unusual practices were used, such as embedding grain seeds in the split ends of cuttings. This unusual technique, used by early European and Middle Eastern propagators, was later found to have a sound scientific basis because germinating seeds produce IAA (HARTMANN and KESTER, 1983). Such techniques were sometimes effective, but they were often unreliable and generally the rooting of cuttings became more art than science.

The concept of a compound with the specific ability to initiate the regeneration of roots was first proposed by SACHS (1880 a, 1880 b). He explained the polar regeneration of roots in terms of a "rhizome" substance, synthesized in the leaves of cuttings and transported basipetally to the region of regeneration. BOUILLENNE and WENT (1933), cited by HESS, 1961, subsequently used the term "rhizocaline" to describe that substance. Rhizocaline was seemingly an acid compound of low molecular weight, heat stable and stored in the cotyledons and buds of *Impatiens balsamina* L. (WENT, 1934). Other factors such as nutrients, carbohydrates, phenolics, nitrogenous compounds, vitamins and inorganics are now known to be involved in root initiation and development (JARVIS, 1986). The unpredictable nature of rooting was removed, or at least reduced, as a result of the chemical identification and elucidation of IAA and other synthetic indoles. It also resulted in the discovery that auxins induce root initiation (THIMANN and WENT, 1934; COOPER, 1935) and that 'rhizocaline" was physiologically similar to auxin (THIMANN and KOEPFLI, 1935). The basipetal transport of this substance was as a result of the auxin gradient established by the polar movement of auxin. This substance had to be present together with IAA to allow root formation. Although rhizocaline has been proposed to be a non-specific carbohydrate or nitrogenous compound and has been thought to be Vitamin K or biotin,

research showed no relationship between the unknown rooting substances and amino acids from casein hydrolysate, yeast vitamins or ammonium sulphate (KAWASE, 1964).

BOUILLENNE and BOUILLENNE-WALRAND (1955) proposed that rhizocaline is the product of a reaction between auxin and an orthodiphenolic, catalyzed by an oxidase enzyme (cited in WILSON, 1988). These three factors were collectively referred to as the "rhizocaline complex". Phenolics promote rooting in some plants (WILSON, 1988). It was hypothesized that orthodiphenolics react directly with auxin to form one or more auxin-phenolic conjugate(s) which create the predisposition to root (HAISSIG, 1974; WILSON, 1994). These discoveries in the history of plant propagation led to auxin treatment of cuttings to promote rooting, and made it possible to consistently root large quantities of cuttings from plants which previously had been very difficult, if not impossible, to propagate vegetatively (BLAZICH, 1988 a).

In order to obtain high-quality young plants in the shortest possible time, cuttings must root quickly and abundantly. Cuttings must also be capable of good lateral branching and fast growth after rooting (MOE and ANDERSEN, 1988). Treatment with growth regulators increases the speed and percentage of rooting, especially with difficult-to-root species (AVERY and JOHNSON, 1947; WRIGHT, 1962; BLAZICH, 1988 a). Growth regulators also help increase the number, quality and uniformity of roots produced per cutting.

The method of growth regulator application is important. Three of the most widely used techniques are the Powder Method, the Soak Method and the Quick-dip Method. The Powder Method first used by GRACE (1939) has been effective with cuttings of a number of different species. In some cases the dust has been prepared by merely grinding the active chemical to a fine powder and then mixing it thoroughly with the dust carrier by shaking. Talc or powdered charcoal have been the favorite carriers (PEARCE, 1948; MACDONALD, 1986).

The Soak Method, first used by COOPER (1935) and HITCHCOCK and ZIMMERMAN (1937) consists of soaking the cuttings for 24 hours, in low concentrations of the hormone solution. The rate of absorption usually depends on the temperature and humidity during the treatment period, as well as light intensity and the density at which the cuttings were packed in the container holding the solution. Under high evaporation conditions, where the leaves transpire rapidly, over dosage and injury are possible.

The Quick-dip Method is not susceptible to as many variable factors during the absorption period as the Soak Method. It also has an advantage over the Powder Method as it allows for more uniform distribution of hormone to the cutting. The Quick-dip method relies on the cuttings having a short exposure of 15 minutes to high hormone concentrations.

Attributed to the fact that plant tissues possess several metabolic mechanisms that remove natural IAA from the growth regulating system, synthetic IBA and NAA have been judged superior exogenous hormones added as root formation promoters (LEOPOLD and KRIEDEMANN, 1975). IBA is more effective than NAA for most species (HITCHCOCK and ZIMMERMAN, 1937). However, a comparison between the two is not simple because many factors influence auxin efficacy. Thus an auxin-talcum powder mixture versus an auxin solution can influence efficacy (HEUNG and M^{C}GUIRE, 1973; BONAMINO and BLAZICH, 1983).

A review of the literature indicates that the application of Kelpak (a seaweed extract), elicited many and varied responses to plant tissues. These include higher yields, increased nutrient uptake, increased adventitious and lateral root formation, changes in plant tissue composition, increased resistance to frost, fungal diseases and insect attack, longer shelf life of fruit and better germination. CROUCH and VAN STADEN, 1990 (Table 5) proposed that an alternative application to the synthetic hormone powders used conventionally in root growth induction on cuttings, is the utilization of Kelpak solution. Kelpak is a seaweed extract manufactured by Kelp Products (Pty) Ltd., Cape Town, Republic of South Africa,

Table 5: Rooting responses of cuttings brought about by the application of Kelpak, a seaweed extract

Plant Species	Seaweed Product	Growth parameters affected	Comments	Reference
Callistemon citrinus (Bottlebrush)	Kelpak (cutting dip) 1: 10	Increased root growth	Initiated > 130% more roots	CROUCH and VAN STADEN, 1990
Dianthus deltoides (Carnation)	Kelpak (cutting dip) 1: 10	Increased root growth	Initiated about 50% more roots than control cuttings.	CROUCH and VAN STADEN, 1990
Evolvulus glomeratus	Kelpak (cutting dip) 1: 10	Increased root growth	Initiated >60% more roots than control cuttings	CROUCH and VAN STADEN, 1990
Impatiens auricoma	Kelpak (cutting dip) 1: 10	Increased root growth	Initiated >100% more roots than control cuttings	CROUCH and VAN STADEN, 1990
Lavendula vera (Lavender)	Kelpak (cutting dip) 1: 10	Increased root growth	Initiated about 18% more roots on cuttings	CROUCH and VAN STADEN, 1990
Vitis agnus-castrus	Kelpak (cutting dip) 1: 10	Increased root growth	Initiated over 300% more roots on cuttings	CROUCH and VAN STADEN, 1990

Constituents	Kelpak (mass per cubic decimeter)
Macro/Micro Nutrients	
Protein	3000mg
Carbohydrates (alginates, laminarin, mannitol)	16 900mg
Nitrogen	
Phosphorus	3600mg
Potassium	8200mg
Barium	7200mg
Boron	1900mg
Calcium	240mg
Cobalt	800 mg
Copper	0.3 mg
Fluorine	0.2 mg
Iodine	0.4 mg
Iron	8.6 mg
Magnesium	13.6 mg
Manganese	200 mg
Molybdenum	8.4 mg
Nickel	0.38 mg
Sodium	0.34 mg
Strontium	800 mg
Sulphur	0.4 mg
Zinc	0.64 mg
	4.2 mg
Vitamins	
B1	
B2	0.08 mg
C	0.08 mg
E	20 mg
	0.68 mg
Amino Acids	
Alanine	
Valine	280 mg
Glycine	150 mg
Isoleucine	140 mg
Leucine	92 mg
Proline	180 mg
Threonine	184 mg
Serine	152 mg
Methionine	208 mg
Hydroxyproline	72 mg
Phenylalanine	36 mg
Aspartic Acid	8 mg
Glutamic Acid	316 mg
Tyrosine	20 mg
Ornithine	332 mg
Lysine	20 mg
Arginine	272 mg
	16 mg
Growth Regulators	
Auxins	
Cytokinins	0.031 mg
Gibberellins	

from the stipes of the brown algae *Ecklonia maxima* (Osbeck) Papenfuss, using a cell burst process. This process does not involve the use of heat, chemicals or dehydration which could affect some of the organic components (Table 6) of the concentrate.

Just like the type of rooting hormone and its' optimum dosages vary for cuttings of different species, the ease with which different varieties of plants can be multiplied by cuttings varies widely. Careful consideration of factors such as:

- the age and condition of the plant;
- the type and physiological condition of the cutting selected;
- the time at which the cuttings are taken; and
- the conditions under which treatment was carried out and cuttings were rooted;

may make all the difference between success and failure (LEAKEY, 1983).

The physiological condition of the stock plant is the result of the interaction between genotype and environmental factors (light, temperature, water, carbon dioxide and nutrition). It is evident that the stock plant environment exerts a strong influence on root formation in stem cuttings (HEIDE, 1964;1965 a and 1965 b). Therefore, after selecting and treating the cutting, careful attention has to be paid to the method of planting, the rooting medium used and the environmental conditions of moisture, temperature and light during the rooting period.

Numerous researchers, using widely different plants, have found that increased root formation after treatment occurs only when the cuttings used are in a certain stage of growth. The rooting capacity of the cutting is generally improved by selecting younger plants and initiating cuttings close to the periphery of the plant (MAHLSTEDE and HABER, 1957; WRIGHT, 1962). Some species react best when the cuttings are taken while in active growth, whereas others react best when growth is slowing down or has ceased. No general rule can therefore be discerned. The best material to use for different varieties and species of plants can only be found by experiment (PEARSE, 1948).

While external conditions may exert a considerable influence on root development in cuttings, the internal conditions are of major importance. Thus, for roots to develop, it is necessary that active meristems should be present in the cuttings, and sufficient nutrients must be present to supply the energy for the development of roots by these meristems (PEARSE, 1939). The majority of stems do not have pre-formed roots, but have the ability to form roots at the base of severed stem pieces. The outer living cells of the cut stem base die immediately after a cutting is made, forming a necrotic plate and the wounded region becomes sealed with suberin. The cells immediately above the necrotic plate divide to form a parenchymatous callus. Thereafter, cells in the region of the vascular cambium and phloem begin to initiate adventitious roots (HARTMANN and KESTER, 1983; LOVELL and WHITE, 1986).

Season dramatically affects rooting in many plants. For most plants, especially deciduous species, cuttings taken in spring or summer root much better than cuttings taken in winter (ANAND and HEBERLEIN, 1975; HARTMANN and KESTER, 1983; THOMPSON, 1986). This could be because the leaves senesce and therefore abscise during the autumn/winter months, thus no longer being able to photosynthesize and provide the required source of nutrients for root production. It has to be kept in mind too that cuttings no longer have access to translocated photosynthates, as they are completely independent of the parent plant. Cuttings made in spring and summer are still able to provide their own nutrients and so are able to regenerate new tissue more easily. Some deciduous hardwood species, do, however, root excellently from winter cuttings with dormant buds (HARTMANN and KESTER, 1983).

The adventitious rooting potential of many woody species, particularly tree species, may decrease during ontogenetic ageing and maturation. The reasons for loss of rooting potential with maturation are very poorly understood, but is believed to be biochemically related (MAHLSTEDE and HABER, 1957). Usually, treatment of cuttings with auxins enhances root initiation in cuttings from juvenile plants, while such treatment enhances cell

division but not root initiation in difficult-to-root cuttings from mature plants. This indicates that rooting of mature cuttings is not limited by endogenous auxin (HACKETT, 1988).

3.2 Materials and Methods

Cuttings were taken at different times during spring, summer and early autumn (explant material taken during late autumn/winter was unsuitable as it was too woody and being a deciduous plant, had entered a stage of dormancy). The choice of branches from which cuttings were made was random, provided the wood was soft to semi-hardwood. To begin with, the 2nd, 3rd and 4th terminal nodes of the growing branches were used separately (Plate 3 a). The 2nd and 3rd nodes were primarily the easier-to-root softwood, whereas the 4th nodes had usually started to become lignified, and accordingly were classified as semi-hardwood. Later, a three node cutting without a split base (Plate 3 b) and with a split base (Plate 3 c) were used.

The cuttings were made in the cool, early morning hours when the stems were still turgid. Shoots were placed loosely in buckets half filled with water immediately after harvest. Shoots were never left in buckets for longer than thirty minutes before cuttings were struck.

Water loss in *Combretum bracteosum* cuttings lead to excessive wilting and ultimately death. In preparing cuttings for treatment, the number of leaves per node retained on the cuttings was limited to two and their area was also reduced by half in order to lower transpiration and to standardize the surface area available for photosynthesis (Plate 3). Where applicable, the apical bud was also removed from the cutting.

Prior to treatment with hormones, the cuttings were soaked in the fungicide, Benlate®, for 5 minutes (concentration 5 g.ℓ^{-1}). The control cuttings were also soaked in the fungicide, but were treated with tap water rather than with hormones during experimentation.

Three methods of hormone application were compared according to cutting growth response.

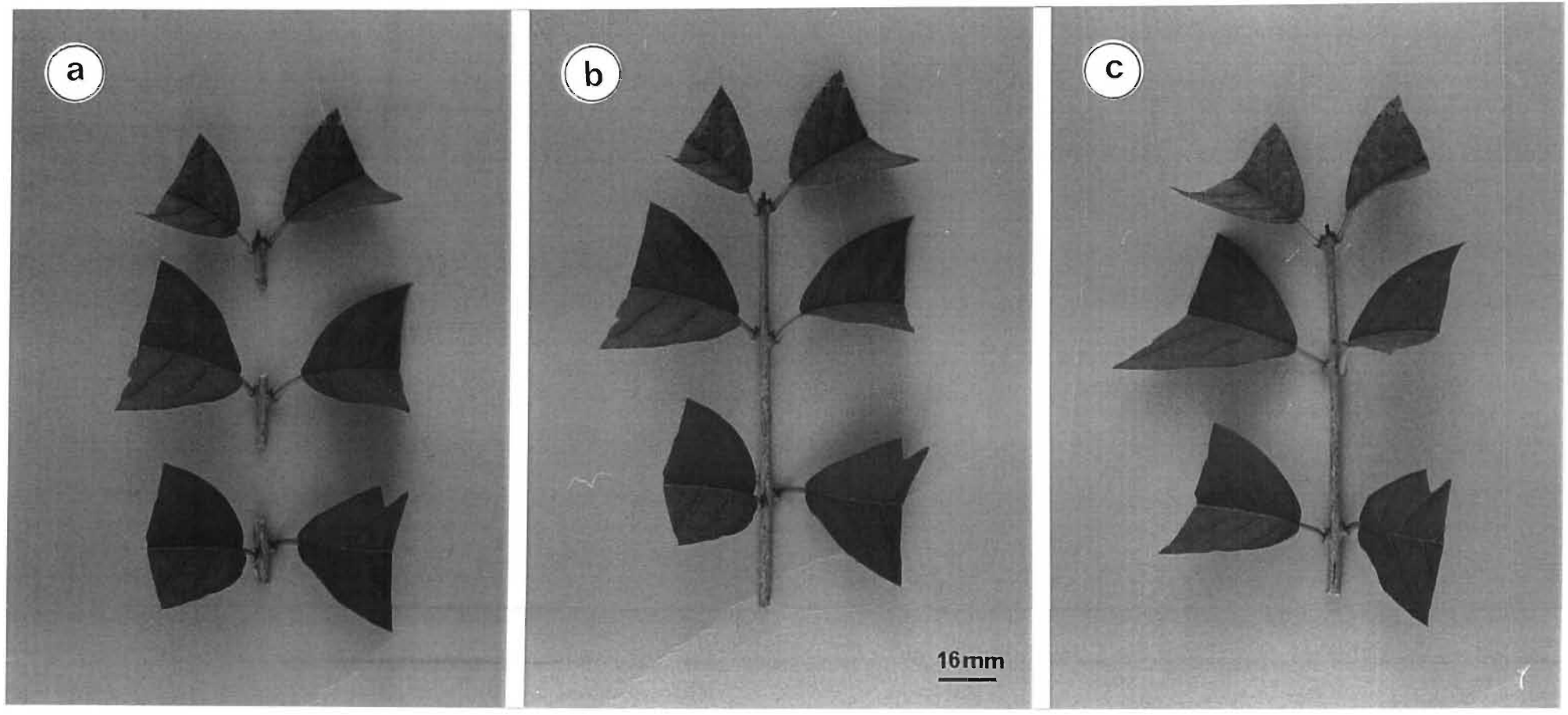


Plate 3: (a) Single node cuttings; and three node cuttings, (b) without and (c) with a split base, struck from a *Combretum bracteosum* shrub

The first was a powder application, where Seradix® 2 (generally used in stimulating root induction of semi-hardwood cuttings), was dusted onto the cut base of the cuttings. These were subsequently placed directly into the potting media.

The other two methods, the soak and quick-dip methods tested the ability of the synthetic root-promoting chemicals indolebutyric acid (IBA) and naphthaleneacetic acid (NAA) in inducing root growth on *Combretum bracteosum* cuttings. A seaweed extract, Kelpak, containing natural plant hormones, was also tested. Immediately after being struck, twenty-five cuttings were placed into a plastic beaker containing a hormone solution. Soak treatment concentrations were relatively low, due to the prolonged exposure (twenty-four hours) of the cuttings to the active ingredient. Hormone concentrations used for this technique were 2, 5, 10, 50, 100 and 250 $mg.\ell^{-1}$ IBA and NAA (alone and in combination). The latter three concentrations were incorporated into the dilution series due to the initial lack of response with the lower three concentrations. Kelpak treatments consisted of 1, 2, 5 and 10% of the concentrated seaweed extract.

The Quick-dip Method allowed the cuttings to be exposed to high hormone concentrations, for short periods of time (15 minutes). Hormone concentrations used for this technique were 50, 100, 250, 500, 1000 and 2500 $mg.\ell^{-1}$ IBA and NAA (alone and in combination). Treatment of cuttings with Kelpak consisted of 0, 25, 50, 75 and 100% of the concentrated seaweed extract.

In making up hormone solutions, ethanol (0% - 70%) was incorporated into the test solution in an attempt to amplify cutting response to exogenous rooting hormone treatments. By stimulating dehydration within the cutting it was hoped that absorption of IBA and NAA would be enhanced. This was not so however as the dehydration led to rapid cutting mortality, resulting in all subsequent hormone solutions being aqueous.

After treatment, the *Combretum bracteosum* cuttings were placed in a seedling trays

containing potting media. The composition of basal media most suitable for root initiation on cuttings was determined. Combinations of 1 part vermiculite, 1 part peat moss and 2 parts Umgeni sand; 2 parts vermiculite, 1 part peat moss and 2 parts Umgeni sand; 1 part vermiculite, 1 part compost and 2 parts Umgeni sand; vermiculite only or perlite only were tested. Media combinations used in further experimentation were perlite only under misthouse conditions, and 1 part vermiculite, 1 part peat moss and 2 parts Umgeni sand under greenhouse conditions. Cuttings were also provided with a soil drench of half strength MURASHIGE and SKOOG (1962) nutrient media immediately after being placed into the potting media. This was repeated thrice at three week intervals.

Two different environments were used to test the rooting ability of the *Combretum* cuttings. One replicate of the experiment was placed in a greenhouse, with no heating but protection from extreme variation of the environment. The other trial was placed in the mist house, which provided basal heating (18-21°C), and environmental protection. The misthouse had a modern intermittent mist system which provided sufficient water to increase the relative humidity of the air around the leaves as well as to reduce leaf and air temperature.

Observations were made weekly and results were obtained as the leaves from the cuttings senesced. The cuttings were then classified according to whether they exhibited no response, callus growth or rooting. A general analysis of variance was used in the statistical analysis of all data. The confidence level was set at 95%. All experiments were repeated at least twice.

3.3 Results and Discussion

The use of cuttings in propagation brings plants to maturity more quickly than from seed and gives uniform sized stocks for planting. The rate of root formation is important since rapid rooting minimizes cutting exposure to adverse environmental factors, and to diseases to which unrooted cuttings are prone (HOWARD, 1994). Hormone-treated cuttings generally root more rapidly and have heavier root systems. There is much variation in the rooting

response of cuttings from different individual cuttings of the same species and also among varieties within a species. Success in rooting depends largely on the physiological condition of the plant from which cuttings are struck. In addition, differences in the age of the plant and the position from which the cutting is taken are factors in rooting that must be considered (AVERY and JOHNSON, 1947).

Prior to striking the cuttings, it is necessary to understand the internal controls of adventitious rooting in order to comprehend both the fundamental developmental biology of rooting and to improve rooting for commercial purposes (HAISSIG *et al.,* 1992). The rooting of vegetatively propagated leafy cuttings involves the complex interaction of many processes (DICK and DEWAR, 1992). The number of physiological and biochemical processes involved in root formation is so large that the factors that regulate the rooting process have been difficult to identify (RIEMENSCHNEIDER, 1994).

Adventitious root formation on cuttings is a function of both a delicate balance between stimulative and inhibitory endogenous factors (WIESMAN and RIOV, 1994) and anatomical conditions existing in the stem. Factors affecting the success of propagation by cuttings include pre-existing conditions of food supply, juvenility and hormone balance (MAHLSTEDE and HABER, 1957). The primary endogenous factors controlling the initiation and development of adventitious roots from cuttings include the carbon, water- and nutrient-status of the cutting, as well as hormonal factors (DICK and DEWAR, 1992). If any one factor is limiting, the whole complex becomes ineffective in triggering root formation (MULLINS, 1972; WRIGHT, 1973). In addition, the effect of the external environment on these endogenous factors, both prior to and subsequent to the detachment from the parent plant, is important. These factors include light intensity, air and soil temperatures and nutrient supply (DICK and DEWAR, 1992).

Root formation involves a sequence of morphogenetic events with differing requirements (MULLINS, 1972). The process of adventitious root formation can be divided into three

stages, namely dedifferentiation, induction and differentiation (DE KLERK *et al.,* 1995). These can be described further as:

1. Cellular dedifferentiation of specific cells, followed by meristematic cell initiation (root initial formation);
2. Development of the root initials into recognizable root primordia (Primordium initiation depends more upon auxin than do subsequent phases of primordial development (HAISSIG, 1986), and together with unidentified factors, auxin is a principle endogenous promoter of root primordium initiation (GAUTHERET, 1969; HAISSIG, 1972)); and
3. Formation of vascular connections with the conducting tissues of the cutting and the rupturing of other stem tissue to allow emergence of the new functioning roots. The initiation of adventitious roots in cuttings is followed by considerable metabolic activity (HARTMANN and KESTER, 1983).

VAN DER LEK (1925) cited in PEARSE (1939) showed that root formation in cuttings of *Salix*, *Populus*, *Ribes* and *Vitis* is largely dependant upon the existence of buds, especially strongly sprouting ones. Roots can be produced by cuttings with all the buds removed, but they are less abundant and smaller than in cuttings with buds present. VAN DER LEK therefore assumed that one or more substances are formed in sprouting buds which are transported in a basal direction through the phloem and stimulate root formation (PEARSE, 1939). In 1934, VAN DER LEK found that with cuttings taken from material which had just become dormant, the buds did not exercise a stimulating influence on root formation, but on the contrary acted more or less as a check which appeared to be correlated with the fact that the buds were not yet in a condition to sprout. As the buds progressively approach the termination of dormancy the checking effect disappears and is replaced by a stimulating influence (PEARSE, 1939; ROWE-DUTTON, 1959).

As the rooting capacity of the cutting is generally improved by selecting younger plants and initiating cuttings close to the plants' periphery (MAHLSTEDE and HABER, 1957), the

older *Combretum bracteosum* shrubs had to be cut back hard prior to experimentation, to initiate the production of suitable cutting material. The much-branched, many stemmed habit of *Combretum bracteosum* was therefore capable of providing large amounts of suitable juvenile plant material for experimental purposes. The choice of cuttings had to be very specific - the extremely fast-growing, soft, tender shoots were not desirable, as they deteriorated prior to rooting, however at the other extreme, older woody stems were slow to root or just dropped their leaves, with no rooting. "Maturing" softwood was chosen for cutting material, from the potentially woody *C. bracteosum* shrub (prior to lignification). The countless axillary buds on these juvenile *Combretum bracteosum* shoots had the potential to begin elongating into lateral branches. It was therefore hypothesized that the endogenous hormone levels present would enable quick and profuse root initiation after the cuttings had been struck. This was nevertheless not the case and exogenous hormone applications seemed a practical alternative.

The rooting response of any cutting is dependent, not only on the age of the stock material from which the cutting is taken, but on the promotory compound used, its concentration, the duration of the treatment and the time interval between excision of the cutting and commencement of treatment (JARVIS, 1986). Although there is no direct evidence of the mode of action of auxins or co-factors in the control or direction of the rooting process, their influence is well known in-that it plays a central role in lateral root and adventitious root formation (PELOSI *et al.*, 1995), they hasten root initiation, increase the number and quality of roots produced per cutting as well as increase the uniformity of rooting (HAISSIG, 1974; CROUCH, 1990). The use of such substances, however, is not a substitute for good propagation practices, such as maintenance of proper water relations, temperature, and light conditions. Although treatment of cuttings with root promoting substances is useful in propagating plants, the ultimate size and vigor of such treated plants is no greater than that obtained with untreated plants (HEIDE, 1965 b).

Indole-butyric acid (IBA), dispensed in powder (talc) or liquid preparations, enhances the

rooting of cuttings, although the liquid IBA formulations are often more effective. This was confirmed by results obtained from attempts to stimulate rooting using Seradix 2, a rooting powder containing IBA. Seradix 2 proved unsuccessful in this regard. A few of the cuttings produced callus. However, this never appeared to a prerequisite for root formation.

As higher levels of endogenous auxin have been related to the initiation of adventitious root primordia (CROUCH, 1990) and to the application of exogenous hormones in solution, it is not surprising that high concentrations ($\geq$ 100 mg.ℓ^{-1} IBA) may induce rooting of many difficult-to-root woody species (CHONG *et al.*, 1992; CHONG and HAMERSMA, 1995). These high concentrations are just below toxicity levels (JACKSON and HARNEY, 1970; MIDDLETON, 1977; HARTMANN and KESTER, 1983; CROUCH, 1990). Too high a concentration of auxin may have a deleterious effect on the number of roots produced, due to the death of the cortical tissue (THIMANN, 1977). Injuries caused by toxic concentrations of hormones are indicated by a yellowing and loss of leaves, inhibition of bud growth, poor callus formation, and a blackening and eventual necrosis of the stem base. Softwood cuttings of deciduous shrubs are more susceptible to injury by high concentrations of hormones than herbaceous plants. Similarly, the very woody species are more readily injured than less woody ones (AVERY and JOHNSON, 1947).

Many different substances which are not closely related chemically are active in root formation, as each particular substance would have its own specific effects. This highlights the case of auxins, where it has been pointed out that qualitative differences exist in roots induced by IBA and $\propto$-naphthalene-acetic acid (NAA). The roots formed under the influence of the former auxin usually appear more normal and well branched, while with the latter auxin, they usually appear fleshy with few branches (PEARSE, 1939). Mixtures of hormones (e.g. IBA and NAA) are documented for other species (AVERY and JOHNSON, 1947; GASPAR and HOFINGER, 1988), and appear to be more effective than equivalent concentrations of a single hormone for rooting cuttings of *Combretum bracteosum*.

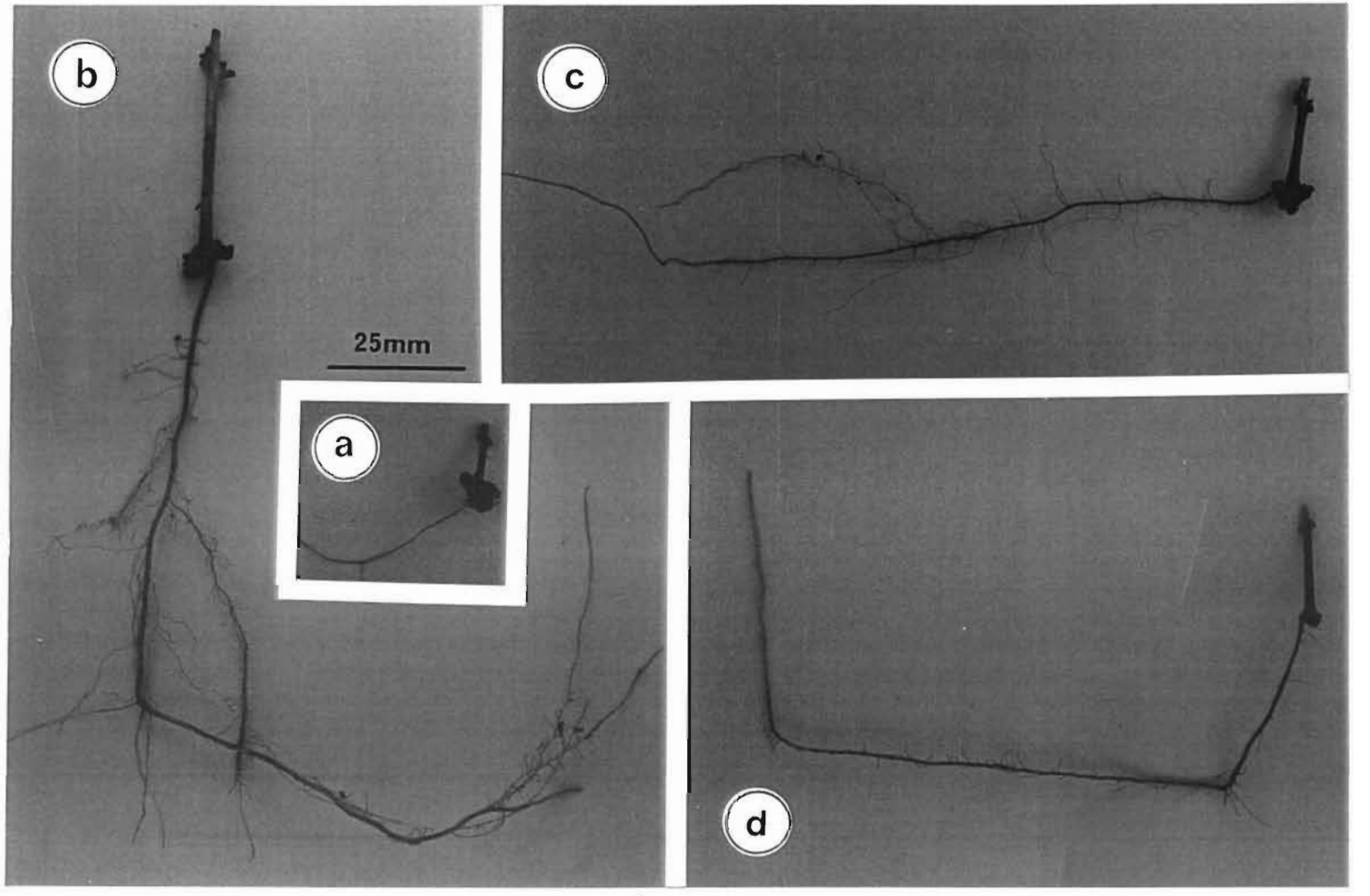


Plate 4: Root formation on single node *Combretum bracteosum* cuttings initiated from a (a) 50 mg.ℓ^{-1} IBA; (b) 100 mg.ℓ^{-1} NAA; (c) 50 mg.ℓ^{-1} NAA; and (d) 100 mg.ℓ^{-1} IBA Quick-dip treatment. All explants represented by single scale bar

Generally, the ability of *Combretum bracteosum* to produce roots, even with the stimulation of a hormone treatment was poor. Initially, individual hormone applications - IBA and NAA - at concentrations of 2, 5 and 10 mg.ℓ^{-1} (Soak Method) and 50, 100 and 250 mg.ℓ^{-1} (Quick-dip) proved unsuccessful. Callus formation within the first two weeks was profuse and characteristic of all treatments. It was evident however, that healthier callus tissue had developed under mist house incubation - the basal heating was believed to be responsible.

Good callus formation does not necessarily lead to good root production. This was highlighted in the case of *C. bracteosum* cuttings, as the callus typically formed what was thought to be root primordia nodules but then died. This callus death was thought to be a direct result of the premature senescence of the cuttings' leaves, where the cuttings were unable to stabilize themselves or cope with the water and photosynthate stress. Callus appeared to develop most readily off cuttings struck from the 4th node, however that was not proceeded by any root development. Ultimately, only a few of the cuttings were able to develop roots (Plate 4). Root formation, although less than 10% in all cases, was best on the Quick-dip NAA-treated 3rd node cuttings (Plate 4 b). The long-dip-treated single node cuttings (2nd, 3rd and 4th nodes) showed no response to both auxins at all concentrations. These random results were not associated with a specific hormone concentration. The adventitious roots that did form, were initiated off the side of the callus, and invariably only a single root was present. These results were unsatisfactory and other treatments had to be sought to induce more reliable results.

At this stage, both single and three node cuttings were struck, with the three-node cuttings having either a split base or an intact base. Numerous alternatives as to the best location for the cut base were also tested. It was only after extensive experimentation by CHADWICK (1931) in ROWE-DUTTON (1959), that it was discovered that while in some plants it is important that a certain type of cut be made, others are not as specific. With most shrubs, the position of the basal cut can vary from the node to half an inch above or below it without influencing the rooting ability of the cutting to any great extent. This is due to the

accumulation of carbohydrates at the nodes and the anatomy of the stem segments (MAHLSTEDE and HABER, 1957). In addition to the energy reserves, preformed adventitious root initials, if present, are usually distributed around the node enabling rapid root regeneration.

Callus and root formation on the three-node cuttings with a split base were superior to the single node cuttings and three-node cuttings without a split base. This method was therefore used in subsequent experiments. The three node cuttings remained alive and responsive to exogenous hormones for 8 - 10 days longer than the single node cuttings, resulting in their response appearing more successful. It was presumed that the success of the single node cuttings especially, would have been far better in all cases had their death rate not been so rapid.

BAYLEY BALFOUR (1913) in ROWE-DUTTON (1959), in discussing whether to remove any of the leaves on the cutting, favored the retention of all the leaves with the view that the cutting is consequently spared the necessity to heal the wounds caused by leaf removal. He also suggested that the lower leaves which are sunk in the rooting medium may form roots like the stem and thereby aid water absorption, while the additional leaves could also aid in the production of food for the cutting (ROWE-DUTTON, 1959). This concept, together with the presence of buds on the cutting to influence rooting, is still common practice today (MAHLSTEDE and HABER, 1957; HARTMANN and KESTER, 1983). Various root inducing factors (rooting co-factors or auxin synergists) are produced in buds and leaves which interact with auxin to promote rooting (HARTMANN and KESTER, 1983). Keeping *Combretum bracteosum* leaves intact on the cuttings as suggested by BELFOUR (1913) was not successful as the leaves turned brown and senesced a week or so after the cutting being struck, irrespective of hormone treatment type or concentration. Reducing the size of the leaf on the cutting by half appeared to delay leaf senescence and subsequent premature death of the cuttings slightly.

In order to minimize the water and nutrient stress imposed on the new cuttings, and so limit their mortality, a spray application of half-strength modified MURASHIGE and SKOOG (1962) or half-strength HOAGLANDS (HOAGLAND and SNYDER, 1933; GEORGE, 1993) nutrient solutions was implemented immediately after the cuttings were struck and repeated every two days. The spray application of both these solutions appeared to hasten leaf senescence. The subsequent application of a soil drench appeared beneficial with the half-strength MURASHIGE and SKOOG (1962) delaying leaf senescence to the greatest extent.

Callus development in the three node cuttings, was healthier and more prolific than had been obtained in previous results, especially with NAA long-dip treatments. At this point it was noticed that although initial root formation was from the callus only, the successive roots were initiated both directly off the wounded base and callus (Plate 5). Most of the roots that were able to establish themselves however, emerged from the callus - this was not ideal.

Researchers generally agree that callus formation and root production are two independent processes, although the two are usually collateral (MAHLSTEDE and HABER, 1957; HARTMANN and KESTER, 1983). Callus usually forms at the basal end of the cutting, as a result of the division of living cells behind the necrotic plate (HARTMANN and KESTER, 1983). This is a proliferation of parenchymatous tissue, formed from young cells in the region of the vascular cambium, although the cells of the cortex, pith and phloem may also be involved. The process of splitting the cutting longitudinally causes increased callus development in the presence of IBA, and enhanced rooting associated with the new cambium formed in the callus (HOWARD *et al.*, 1983).

As these results were not satisfactory for commercial purposes, alternative treatments had to be sought. It was postulated that perhaps the poor rooting response was a result of the hormone concentrations being too low. Further experimentation therefore, followed the same methodology with the addition of higher IBA and NAA concentrations. The potting

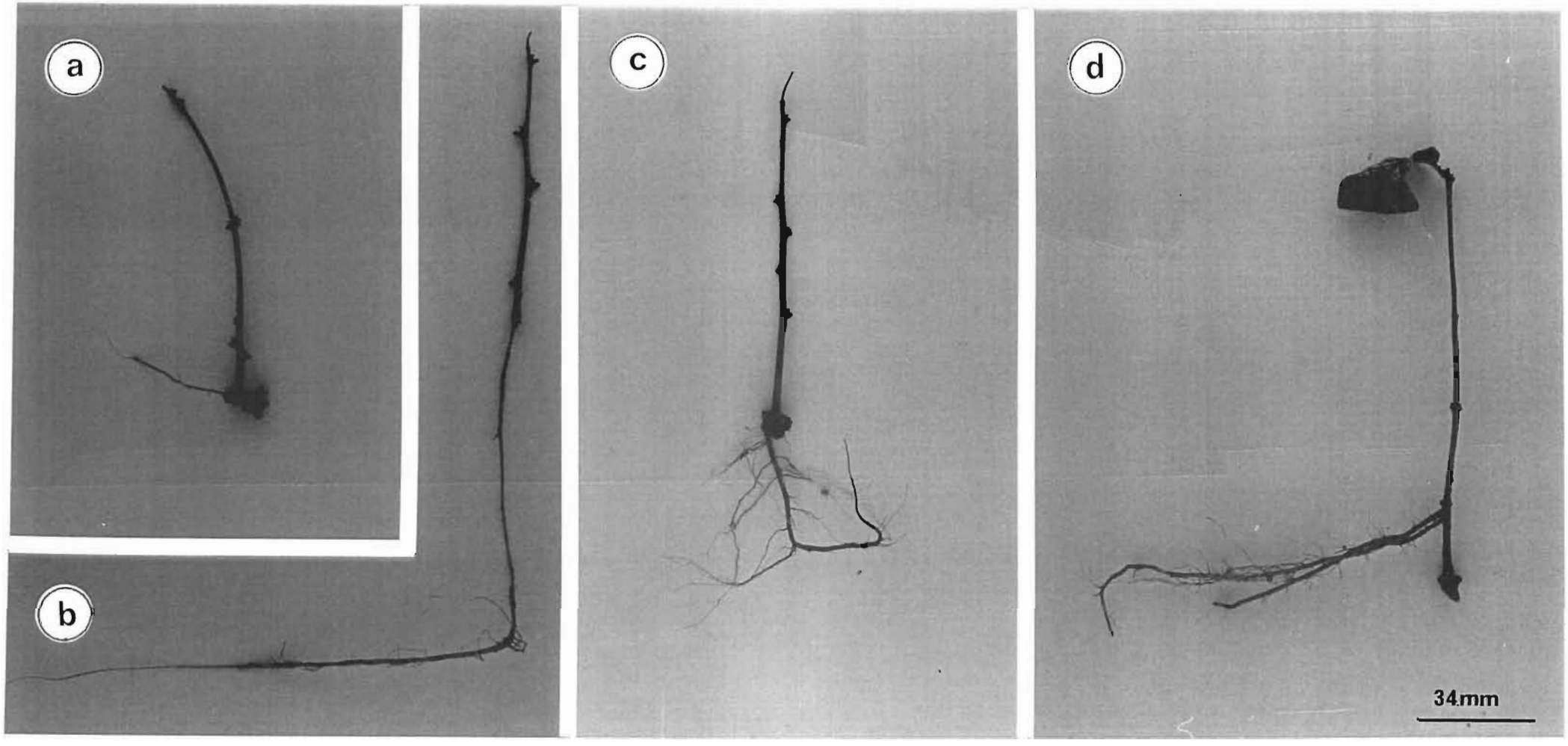


Plate 5: Root formation on three-node *Combretum bracteosum* cuttings stimulated by long-dip treatments (a) 2 mg.ℓ^{-1} NAA; (b) 5 mg.ℓ^{-1} NAA; (c) 2 mg.ℓ^{-1} IBA; (d) 5 mg.ℓ^{-1} IBA. All explants represented by single scale bar

medium also appeared to retain too much water, adding to the chances of premature leaf abscission and basal stem rot. The potting mix should fulfill three main functions, namely to:

- Physically support the cutting;
- Provide moisture for the cutting; and
- Permit drainage of water and penetration of air to the base of the cutting; as advised by HARTMANN and KESTER (1983). Perlite seemed to be a satisfactory alternative in the greenhouse, but in the misthouse, moss growth became a problem. Both perlite and the original potting mix (containing less vermiculite) were used in the greenhouse, whereas, only the original media (containing less vermiculite) were used in the misthouse for subsequent experimentation.

Higher hormone levels were not the solution. However, the change in the potting medium showed promise. The best results were obtained with a long-dip treatment in 5 mg.ℓ^{-1} IBA solution (incubated in the misthouse), which resulted in 40% of the cuttings forming callus and almost the equivalent rooting (Plate 5 d). The reduction in callus formation and the development of two roots directly from the cutting and not off the callus (Plate 5 a and c), made this the most successful result obtained at this point. Although a number of other hormone treatments resulted in root growth, they were not as successful. At this point, it is important to record that the success of *Combretum bracteosum* cuttings seem to be highly dependant on seasonal variation. The lower hormone concentrations which showed less than a 5% response in callus formation (and no root induction five months previously) had increased eight-fold. This large difference could not be attributed to the change in potting media alone. However when considering that the only other varied criteria was the ambient temperature and day-length, seasonal influences on the plant material prior to cuttings being struck could not be ruled out. The effects of seasonal variation will be dealt with later.

It was the success cited in the literature that led to the seaweed concentrate "Kelpak" being used as a cutting treatment. A remarkable feature of several seaweed studies is that

commercial seaweed products significantly increased root growth (FEATONBY-SMITH and VAN STADEN, 1984; FINNIE and VAN STADEN, 1985; BECKETT and VAN STADEN, 1989). Marine algae also contain all major and minor plant nutrients (STEPHENSON, 1968) and trace elements (BOOTH, 1964; YAMAMOTO and ISHIBASHI, 1972; YAMAMOTO *et al*, 1979). In view of the fact that seaweed contains these mineral elements, their possible involvement in the observed growth responses cannot be disregarded. However, the presence of these inorganic constituents as a possible explanation for improved plant growth is not adequate as the amount of seaweed applied to the plants contain too few of these elements to elicit the beneficial responses that have been observed (BLUNDEN, 1977).

Many of the growth effects elicited by seaweed concentrates were similar in nature to those which might be expected to be produced by cytokinin activity. The applications of low cytokinin concentrations (10^{-8} M) has a slight stimulative effect with regard to root initiation (HEIDE, 1965 b; OKORO and GRACE, 1978; WIGHTMAN *et al.,* 1980). This relates to suggestions that a particular stage of initiation and a certain minimum level of cytokinin is essential for the induction of adventitious rooting (ERIKSEN, 1974; HARTMANN and KESTER, 1983). In the intact plant the continuous transport in the transpiration stream of cytokinins synthesized in the root would prevent adventitious root formation. The cessation of this supply due to the removal of the roots would result in a lower concentration of cytokinins and subsequent root initiation (BOLLMARK and ELIASSON, 1986).

However, moderately high levels of cytokinins (10^{-5} M), which could have been present in 100% Kelpak solutions are generally considered to inhibit early stages of rooting (ERIKSEN, 1974; STENLID, 1982; BOLLMARK and ELIASSON, 1986; VAN STADEN and HARTY, 1988). This is not counteracted even by high concentrations of auxin. The loss of the inhibitory effect of cytokinin during the latter phase of initiation, suggests that at this stage, developing root primordia are capable of controlling the level of active cytokinin and thus do not react to the exogenous application of cytokinin (ERIKSEN, 1974).

It was suggested that cytokinins are essential growth substances in this later part of the initiation phase, and that partially initiated root primordia can synthesize cytokinins (ERIKSEN, 1974). Auxin had no effect at this stage (i.e. when root primordia develop into roots).

It was proposed by BATTEN and GOODWIN (1978) that cuttings that do not respond to auxin, lack sufficient endogenous cytokinins for adventitious root formation. This could explain the poor or complete lack of root system development in auxin-treated *Combretum bracteosum* cuttings whereas, cuttings treated with Kelpak (containing auxins and cytokinins) developed more substantial root systems. Auxin and cytokinin are essential for the competence and determination of tissue explants to form roots (ERIKSEN, 1974; MOHNEN, 1994).

The literature referred to in the preceding paragraph pointed towards cutting responses with the application of hormone combinations rather than individual treatments. Further experimentation using hormone combinations was limited to two auxins. The results from these were compared with results obtained from cuttings treated with Kelpak (containing auxins (SCHIEWER and LIBBERT, 1965; SCHIEWER, 1967; AUGIER, 1976 a; WILLIAMS, *et al.*, 1976; SUMERA and CAJIPE, 1981; KINGMAN and MOORE, 1982; SANDERSON and JAMESON, 1986; CROUCH, 1990; CROUCH and VAN STADEN, 1990), cytokinins (BENTLEY-MOWAT and REID, 1968; HUSSIAN and BONEY, 1969; JENNINGS, 1969; AUGIER, 1972; AUGIER and HARADA, 1972; 1973; BRAIN, *et al.*, 1973; VAN STADEN and BREEN, 1973; AUGIER, 1974 a; 1974 b; MOONEY and VAN STADEN; 1986, 1987; CROUCH, 1990; CROUCH and VAN STADEN, 1990), gibberellins (BENTLY, 1960; RADLEY, 1961; KATO, *et al.*, 1962; MOWAT, 1963; 1964; 1965; JENNINGS and McCOMB, 1967; JENNINGS, 1968; STEPHENSON, 1968; GUPTA and SHUKLA, 1969; JENSEN, 1969; HUSSAIN and BONEY, 1973; AUGIER, 1976 b; TAYLOR and WILKINSON, 1977; WILDGOOSE, *et al.*, 1978; GOPALA, 1984) as well as essential macro- and micronutrients). Auxin combinations tested were NAA and IBA

solutions at 50, 100 and 250 mg.ℓ^{-1} (Quick-dip) and 2, 5 and 10 mg.ℓ^{-1} (Soak Method).

In most instances, callus formation at the base of the stem was increased with the auxin combination application. Unlike experiments using the single hormone application, callus formation did not appear to restrict subsequent root formation. All soak treatments displayed root induction with the number and volume of the root system increasing with hormone concentration (Figure 9 b and Plate 6 a - c). The only Quick-dip treatment to display positive results was the 50 mg.ℓ^{-1} IBA: NAA combination (Figure 9 a and Plate 6 d). Higher concentrations of 100 and 250 mg.ℓ^{-1} IBA: NAA proved too strong, with the cuttings going yellow and dying within ten days. The control cuttings also began showing signs of stress (thought to be water related). However none of the other cuttings (50 mg.ℓ^{-1} NAA: IBA quick-dip and all soak-method cuttings) showed the same response. Perhaps water relations of the cuttings had been improved by the NAA: IBA treatments.

Although all the Soak treatments and 50 mg.ℓ^{-1} Quick-dip treatment produced roots, it was the 10 mg.ℓ^{-1} (Soak) and 50 mg.ℓ^{-1} (Quick-dip) that provided the best quality roots (Figure 9). Each replicate (cutting) in both cases produced three or more roots from the cut base. These roots elongated rapidly (10 cm in five weeks) and also displayed a dense development of secondary roots.

The *Combretum bracteosum* cuttings were found to be far more responsive to the application of auxins NAA and IBA in combination rather than individually, with a 10 mg.ℓ^{-1} each NAA: IBA soak treatment recommended best for propagation using synthetic hormones.

The Kelpak-treated cuttings responded well to both the Soak and the Quick-dip treatments. The mortality rate of the cuttings was reduced to below fifty percent, and of the cuttings that survived the initial stress, no less than forty-seven percent formed roots at their cut bases (Figure 10). The poorest response of 47% rooting was with the 1% Kelpak Soak Treatment.

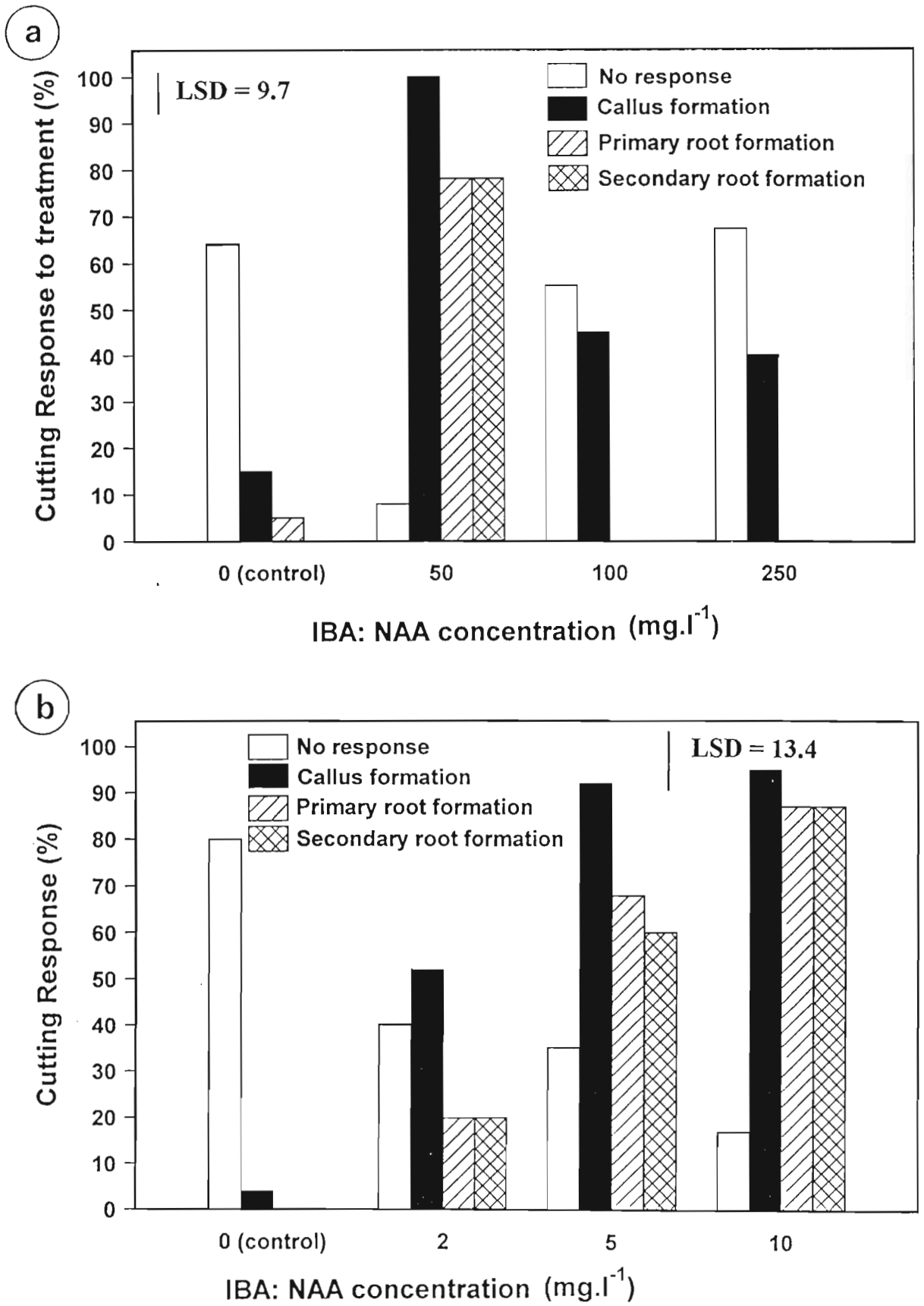


Figure 9: Response of *Combretum bracteosum* cuttings to IBA: NAA combination treatments (a) Quick-dip treatment; (b) Soak treatment. Callus and root development values calculated using "responding" explants only

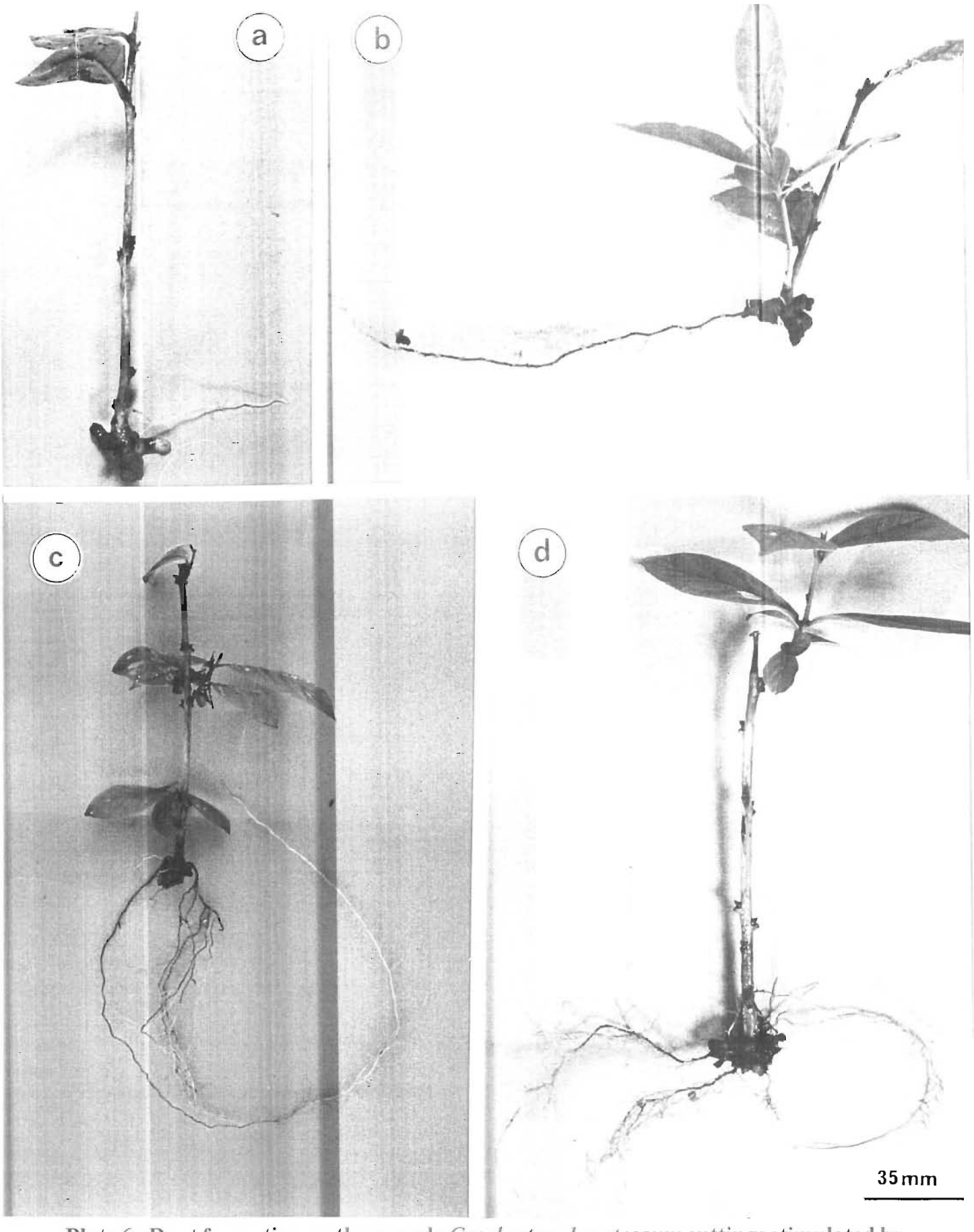


Plate 6: Root formation on three-node *Combretum bracteosum* cuttings stimulated by treatments with varying levels of an IBA: NAA combination (a) 2 mg.ℓ^{-1} Soak treatment; (b) 5 mg.ℓ^{-1} Soak treatment; (c) 10 mg.ℓ^{-1} Soak treatment; (d) 50 mg.ℓ^{-1} Quick-dip treatment. All explants represented by single scale bar

A great deal of energy went into callus formation with only a single small root developing subsequently. This root developed off the callus. The most successful treatment in terms of root formation was initiated by the 50% and 75% Kelpak Quick-dip treatments (Plate 7 a and b). In both cases, callus formation was not profuse and the induction of multiple adventitious roots was directly from the stem base. The root system, even at this early stage of development, was healthy and able to provide the cutting with good anchorage in the potting medium, as well as an unlimited supply of water and nutrients. The cuttings were thriving to the extent that they were able to encourage bud break and exhibit new vegetative shoot formation within eight weeks of being struck.

The full strength Kelpak solution appeared to be above the optimal concentration (Figure 10 a). The cuttings, although forming a competent root system, were not able to revive their growth vigour as rapidly as the 50% and 75% (of the original seaweed concentrate) treatments. It appears that the 100% Kelpak treatment was too high, causing the cuttings to go into a state of shock. However, in metabolising the lethal concentration of compounds the cutting was exposed to, the root initials were presumably reactivated and so were able to continue in developing a root system to support the cutting. Eleven weeks after being struck, cuttings treated with full strength Kelpak concentrate displayed the same morphology as the cuttings treated with 50% and 75% of the concentrated Kelpak solution.

Combretum bracteosum cuttings treated with 1, 2, 5 and 10% Kelpak, appeared to spend a lot of energy producing callus. The root systems that developed were not as strong and substantial as those generated by Quick-dip treatments. All treatments displayed root formation off the callus (Figure 10). Characteristically, the single root that developed (about four centimetres in length) displayed no lateral branching (similar in appearance to roots shown in Plate 6 a). After approximately eight weeks the short root elongated and numerous secondary roots began forming (Plate 7). Cuttings treated with 10% Kelpak were able to initiate a few more primary roots (no longer developing from the callus), which in turn enabled them to encourage more vigorous vegetative growth a few weeks later.

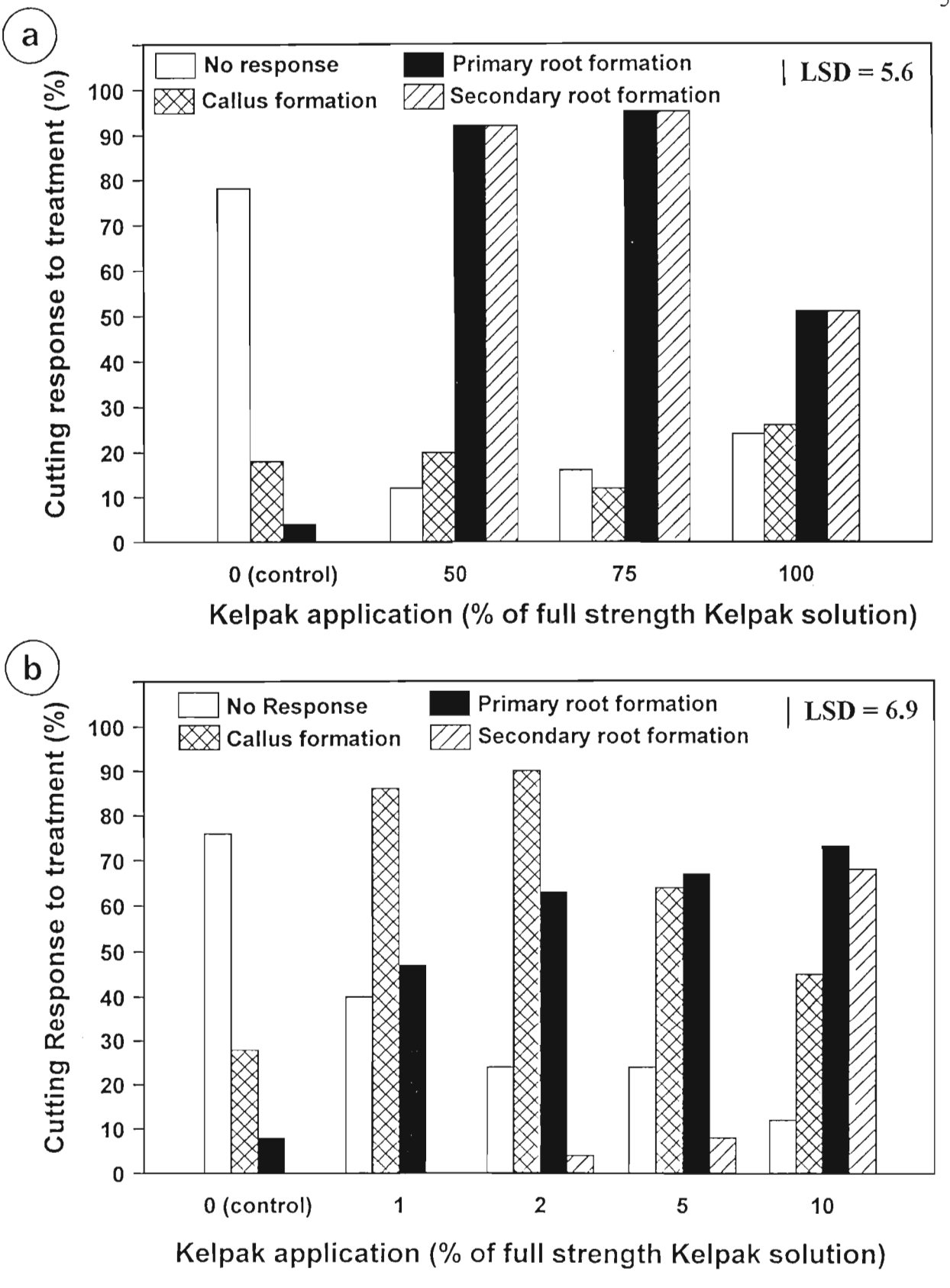


Figure 10: Root development on *Combretum bracteosum* cuttings in response to Kelpak treatments (a) Quick-dip treatment; (b) Soak treatment. Callus and root development values calculated using "Responding" explants only

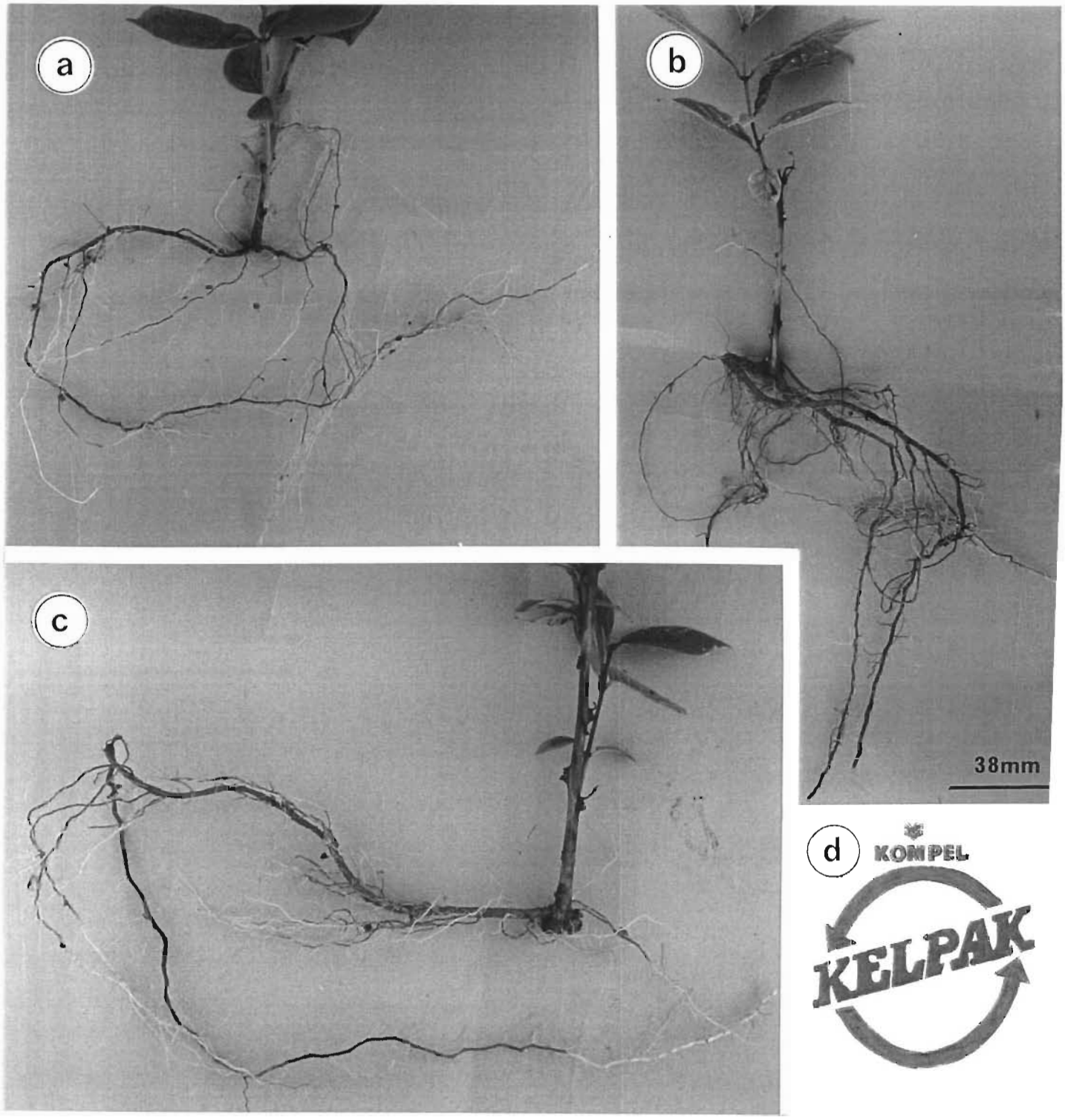


Plate 7: Root formation on three-node *Combretum bracteosum* cuttings treated with Kelpak (a) Quick-dip in 50% Kelpak solution; (b) Quick-dip in 75% Kelpak solution; (c) Soak in 10% Kelpak solution; (d) Kelpak label. All explants represented by similar scale bars

Treatment of *Combretum bracteosum* cuttings with Kelpak was carried out on different occasions, from the second week in September (spring) through to the end of March (autumn), as previous experimentation indicated that seasonal variation impacted on their ability to survive and to produce roots. This phenomenon seems to be related to seasonal variation in the nutritional status of the explant, temperature, irradiance levels, and/or an interaction between irradiance and photoperiod (MOE and ANDERSEN, 1988). Photoperiods that induce dormancy, promote adventitious bud formation or promote flowering in stock plants, generally inhibit or delay rooting of cuttings. Results obtained from cuttings struck during late summer/early autumn were the most successful, with those taken during early spring providing no result as they died off within two weeks of the cuttings being struck. This was predictable as *Combretum bracteosum* shrubs begin flowering in September. Cuttings taken from the vegetative portion of the shrub were depleted of reserve nutrients due to energy and nutrient stores being channeled towards the reproductive phase of growth. Cuttings struck at this time were unable to survive the stress of detachment from the parent plant as the nutritional reserves were assumed to be low and the plant material was still too young to provide sufficient photosynthates for survival on their own.

Literature states that summer is the most appropriate time to induce rooting on stem cuttings (HARTMANN and KESTER, 1983). Not only is the higher ambient temperatures in summer ideal for encouraging root production, but at this time most of the still immature branches that have produced new shoots would have almost grown fully. Their capacity for renewed regenerative growth, however, is still barely suppressed. Cuttings taken during mid-summer were also unsuccessful as temperatures during this period generally ranged from 33 to 40°C. These extreme environmental conditions, although avoided at the time of striking the cuttings, exerted too much stress on the detached cuttings, resulting in them losing their leaves and dying. Cuttings struck during March were the most successful as the temperatures were low enough for the water status within the plant tissue to be sufficient and stable. The plant was no longer flowering and, being deciduous, the movement of

photosynthetic assimilates was no longer toward meristematic regions for developmental purposes, but rather away from the leaves and into the more mature stems and roots for storage.

Other factors which influence the rooting ability of the cuttings are the environmental factors, namely light, temperature, O_2 and CO_2 levels, carbohydrates and the mineral status of the parent plant and cutting, to mention just a few.

It is difficult to separate the effect of temperature changes from a number of other environmental influences (MOE and ANDERSEN, 1988). However, the temperature at which stock plants, and cuttings are maintained, influences the metabolism during rooting. VEIERSKOV *et al.* (1982) suggested that the growth temperature of the stock plant, rather than photosynthesis, was most important in determining the initial carbohydrate content of cuttings. *Combretum bracteosum* cuttings kept in the greenhouse were exposed to different environmental temperatures with weather fluctuations and seasonal changes. Although the cuttings incubated in the misthouse were under regulated temperatures, the beds on which the potting trays were placed were heated. The influential effect of temperature on rooting is thought to be brought about by the translocation of supportive and inhibitory factors, and also the stimulation of mitosis in the rooting zone. Carbohydrates may be one of the supportive factors because the accumulation-metabolism of carbohydrate within pea cuttings has been found to depend on temperature (HAISSIG, 1986).

DYKEMAN (1976) in HAISSIG (1986) tested the rooting of *Chrysanthemum* and *Forsythia* cuttings at 25°C and 30°C. More rapid rooting and more roots per cutting were obtained at 30°C, but root elongation, root diameter and root hair development were superior at 25°C. Thus, higher temperatures favored primordium initiation whereas lower temperatures favored root development. The beneficial influence of higher temperature on initiation may be due to the related increase in respiration and catabolism of simple sugars that would have been stored in starch at lower temperatures (HAISSIG, 1986).

Apparently, the demands for energy and carbon skeletons to support rooting vary between species and depend somewhat on the type of cutting. For example, woody cuttings may require weeks or months to root during which time they can produce large amounts of callus and undergo a substantial increase in mass. In contrast, many herbaceous cuttings initiate primordia quickly, with little increase of mass. Nevertheless, all types of cuttings may be in an equally tenuous energy state when first severed from the stock plant root system. Carbohydrates are considered to be the principal source of energy and yield of carbon skeletons needed for the production of new tissues, during rooting (VEIERSKOV, 1988). This is assumed, as in most cuttings carbohydrates are present in greater concentrations than alternative energy sources such as lipids. This means that a specific threshold level of carbohydrates, before and during experimentation, is important to support growth and development. In part, carbohydrate concentrations in cuttings may be influenced by auxin treatment, which can enhance mobilization of carbohydrates in leaves and the upper stem, and increase transport to the rooting zone (HAISSIG, 1986). By supplementing low carbohydrate levels, the rooting process will be promoted.

Mineral nutrition is another of the many factors which influence adventitious rooting in cuttings. After separation from the stock plant, cuttings have a fixed mineral nutrient pool, except for any minerals absorbed from the rooting medium or irrigation solution. Although adventitious rooting and mineral nutrition are intimately related, the subject is difficult to deal with because root formation on stem cuttings is a multi-stage process and few studies have distinguished between mineral effects at the various stages (HARTMANN and KESTER, 1983). Limited literature evidence suggests that root primordium initiation is not markedly influenced by excesses or deficiencies of any particular mineral nutrient, with the possible exception of nitrogen (STUART, 1938; STRYDOM and HARTMANN, 1960), potassium (GOOD and TUKEY, 1967) and boron (BLAZICH, 1988 b). In order to eliminate the deficiency of any one of these elements resulting in poor, or no root development, an application of half-strength MURASHIGE and SKOOG (1962) solution was necessary. This should have provided the cuttings with the most essential minerals

required by a plant for healthy growth and development. One of the most important of these minerals is nitrogen as it plays a pivotal role in nucleic acid and protein synthesis. It could be argued therefore, that root initiation is primarily influenced by the nutrient contents within the base of a cutting before and during rooting (GOOD and TUKEY, 1967; HAISSIG, 1986; BLAZICH, 1988 b; MOE and ANDERSEN, 1988) and the nutritional status of the stock plant is more important in terms of root growth and development. The absence of redistribution during root initiation may partially explain the reason why the role of particular mineral nutrients in root initiation is so uncertain (BLAZICH, 1988 b).

A discussion of the role of various mineral nutrients in root initiation would not be complete without mention of zinc (Zn) and manganese (Mn), both of which influence endogenous auxin levels. The former being required for the production (TSUI, 1948; SALAMI and KENEFICK, 1970) of the auxin precursor tryptophan (THIMANN, 1935; GOODWIN and MERCER, 1983) and the latter acting as an activator of IAA-oxidase which destroys native auxin (THOMASZEWSKI and THIMANN, 1966). The relationship, however, between Zn, tryptophan, and IAA is not simple because some data suggest that Zn is required for the synthesis of IAA from tryptophan (TAKAKI and KUSHIZAKI, 1970). Due to tryptophan being a precursor of auxin, one would assume poor rooting to result from cuttings taken from Zn deficient stock plants. Critical Zn tissue levels that might influence rooting are not well defined. Studies by SAMISH and SPIEGEL (1958) in BLAZICH (1988 b) however, showed that Zn fertilization of the stock plants improved the quality of the resulting plants as well as increasing tryptophan levels.

Improved mineral nutrition in plants using commercial seaweed preparations is well documented (FRANCKI, 1960 a, 1960 b; AITKEN and SENN, 1965; OFFERMANS, 1968; LYNN, 1972). An increased root system, with its larger surface area available for mineral absorption, is important, but other ways by which seaweed extracts promote mineral nutrition have been suggested. Research suggests that certain constituents in seaweed may play a role in the chelation of metals to give soluble complexes, thus increasing uptake of

trace elements by plants (SENN and KINGMAN, 1978). It is also possible that applied hormones within the extract may act directly on the uptake mechanisms in the roots. It was assumed from these facts on intact plants, that the response of Kelpak-treated cuttings would be similar.

Early studies suggested that mineral nutrients were leached from cuttings during mist propagation (EVANS, 1951; SHARPE, 1955; ROWE-DUTTON, 1959). These findings were later confirmed by more detailed studies which demonstrated that mineral nutrients such as N, P, K, Ca and Mg are leached from cuttings while under mist (GOOD and TUKEY, 1966; BLAZICH *et al*, 1983). Greater leaching from hardwood cuttings has also been attributed to an increased proportion of the nutrients being in an exchangeable form, whereas in young, growing tissues nutrients are quickly metabolized within cells and cell walls which are difficult to leach (GOOD and TUKEY, 1966). In addition to tissue maturity, there are also other factors which influence this phenomenon, such as relative leachability of a particular nutrient (TUKEY *et al*, 1958). This may explain why leaves on cuttings often show signs of mineral nutrient deficiency. The amount of leaching will, of course, depend on the rate of application of water. These problems can be partially overcome by the use of intermittent mist. In an attempt to counteract leaching which occurs under the mist spray, however, a few researchers tried applying controlled release fertilizers either to medium/ foliage through mist spray (ROWE-DUTTON, 1959). Despite enhancement of overall root quality (water roots vs. feeder roots) and subsequent cutting growth (JOHNSON and HAMILTON, 1977; WARD and WHITCOMB, 1979) percentage rooting in numerous instances was unaffected, i.e. supplemented nutrition during rooting has little or no promotive effect on root initiation. This may be related to the inability of unrooted cuttings to absorb nutrients (BLAZICH, 1988 b).

Adventitious rooting often occurs under conditions of water stress in cuttings and in the aerial organs of intact plants. The cause-effect relations between water stress and rooting is partly due to changes in carbohydrate metabolism (ORTON, 1979; RAJAGOPAL and

ANDERSEN, 1980 a; 1980 b). The most immediately evident effect of water stress on the cutting, is closure of the stomata. Stomatal closure affects carbohydrate gain through photosynthesis directly by reducing diffusion of carbon dioxide to the chloroplast, and indirectly by causing a rise in leaf temperature. The direct effect is probably more important, but in most propagation conditions, the bases of cuttings show an appreciable gain in dry weight preceding root initiation. It seems unlikely therefore, that in woody cuttings, carbohydrate supply commonly limits initiation of primordia. However, once roots are initiated, their further growth is heavily dependant upon a good supply of photosynthates (LOACH, 1988).

In general, the normal physiological activities of higher plants, such as root growth, require oxygen. Oxygen influences the biochemistry of mitosis, which is a primary event in root primordium initiation and development. AMOORE (1961) in HAISSIG (1986), suggested that mitosis is less sensitive to O_2 deficiency than root elongation or respiration. AMOORE'S findings indicate that the initiation of root primordia in cuttings is partly controlled by O_2 concentration within the rooting zone, which is usually a solid or liquid medium. Successful rooting may therefore depend on the rate at which O_2 moves to the roots through gas-filled, interconnected pores of the rooting media (HAISSIG, 1986). This reiterates the importance of the potting media used. The original potting media, consisting of vermiculite (to increase porosity and aeration), peat moss and Umgeni sand, appeared to retain too much water in the misthouse, becoming a heavy, solid mass, resulting in the root primordia being surrounded by an anaerobic environment. This combination was however well suited for cuttings in the greenhouse, where a slight water retention in the soil between watering, prevented the cutting from drying out completely. Increasing the proportion of vermiculite in the misthouse potting media from 1 part vermiculte: 1 part peat moss: 2 Umgeni sand, to 2: 1: 2 volumetric parts respectively, resulted in the media becoming slimy, soggy and again presumably anaerobic to root primordia. The aeration and water retention problems were eliminated with the use of Perlite alone as the potting medium. The Perlite granules remained solid (unlike vermiculite which when wet became compact), so keeping

the rooting environment porous for efficient gaseous exchange and continuous flow of excess water out of the potting media, eliminating the water-logging problem.

3.4 Conclusion

Although not the most efficient technique time-wise, stem cuttings appear to have potential in regenerating this beautiful indigenous shrub. The initial lack of success in experimentation by no means indicated that this method is not a viable option, but rather eliminated a number of variables in the tedious process of determining the optimal conditions for this type of propagation. It was established that seasonal variation is an important consideration to make when striking cuttings. The use of Kelpak was the most promising treatment, with the rapid development of roots, and subsequent vegetative growth. The root systems were strong and robust. This has promise for future commercial purposes.

Chapter 4
Micropropagation of *Combretum bracteosum*

4.1 Introduction

Efficient vegetative propagation is essential for the breeding and exploitation of most heterozygous crops. Tissue culture methods have advanced considerably in recent years thus greatly increasing the scope and potential of propagation by exploiting regenerative behaviour more efficiently and in a wider range of plants than is possible with conventional procedures. In-so-doing, establishing the technique in the repertoire of biological techniques. Originally, organs and tissues were cultured in order to study fundamental problems of plant morphogenesis. However, it has become increasingly clear that such cultures, grown under precisely controlled conditions and in the absence of contaminant micro-organisms, provide excellent experimental materials in many other aspects of plant biology.

Micropropagation of ornamentals is the most widely used tissue culture technique. Increasingly, examples are available where micropropagated material is not used as the final product, but rather as micro-mother plants for cutting production. Although axillary budding and single node explants are the most popular ways of propagation, the more complicated technique of meristem culture was one of the first and is still a widely used procedure in commercial applications of tissue culture in ornamentals. MOREL and MARTIN (1952) were the first to develop this technique for *Dahlia*. Later it became a routine operation for many ornamentals, especially cut flowers. The most important applications and the popularity of tissue culture stem from its ability to eradicate viruses.

4.1.1 Germination *in vitro*

In anticipation of decontamination problems *in vitro*, an alternative explant source had to be sought in order to provide the technique of tissue culture with the maximum opportunity

for success. Germinating *Combretum bracteosum* seed *in vitro* could supply sterile explants for later use in axillary shoot elongation and meristem culture.

4.1.2 Axillary shoot elongation

Axillary shoot elongation occurs when inactive axillary buds are released from apical dominance, mostly by hormone manipulation (primarily cytokinins) in the nutrient medium. This method of propagation is more common with hardwood species as it is generally the easiest method available and it maintains genetic stability better than propagation by organogenesis (McCOWN and McCOWN, 1987).

4.1.3 Meristem culture

Most of the crop plants, especially those propagated routinely by vegetative means, are at severe risk of passing on one or more systemic bacterial, fungal or viral infections during the propagation process. Pathogen distribution in plants is uneven, and their presence does not always lead to the plants' death. Many viruses may not even show visible symptoms, however, the presence of viruses in the plant can reduce the yield and/or quality of crops (WANG and HU, 1980). Eradication of viruses and other pathogens is thus highly desirable to optimize the yields and also to facilitate the movement of living plant materials across international boundaries (BUTTON, 1977; BHOHWANI and RAZDAN, 1983).

While plants infected with bacteria and fungi may respond to treatments with bactericidal and fungicidal compounds, there is no commercially available treatment to cure virus-infected plants as most of the virus inhibitors are toxic to the plant (BHOHWANI and RAZDAN, 1983). Killing vectors, such as insects, nematodes and mites may alleviate the spread of certain virus diseases. However, they cannot eliminate the virus from the whole plant. When the treatment ceases, the virus soon recovers to its former concentration. Some viruses, however, are spread mechanically (HU and WANG, 1983; WARREN, 1991), others are stylet-borne, which means that they are transmitted immediately the insect starts feeding. Such viruses cannot be controlled by pesticides. Numerous cases have subsequently been

highlighted where viruses are eradicated from the meristem[1] (shown to be infected at the time of culturing), for example from *Dieffenbachia* (KNAUSS, 1976) and *Pelargonium* (BEAUCHESNE *et al.*, 1977). MELLOR and STACE-SMITH (1977) suggested that such *in vivo* eradication is caused by metabolic disruption resulting from cell injury during the excision process; the smaller the tip excised, the greater the injury and resultant disruption. It seems probable that such *in vivo* virus eradication is more likely to occur if small rather than large amounts of virus are present in the tip (WALKEY, 1980).

In infected plants the apical meristem is generally either free or carry a very low concentration of the virus depending on the type of virus and the host species (MORI, 1977; WANG and HU, 1980). In older tissues the number of viruses increases with increasing distance from the meristem tips. The reasons advanced for the meristem escaping virus invasion include:

(a) viruses readily move in a plant body through the vascular system, which is absent in the meristem, therefore eliminating the phloem- and xylem- borne viruses (WARREN, 1991). The alternative method of cell-to-cell movement of the virus through plasmodesmata is too slow to keep pace with the actively growing tip;

(b) high metabolic activity in the actively dividing meristematic cells do not allow for virus replication (MELLOR and STACE-SMITH, 1977; WANG and HU, 1980);

(c) the "virus inactivating system" in the plant body, if any, has higher activity in the meristem than in any other region. Thus, the meristem is protected from infection; and,

(d) a high endogenous auxin level in shoot apices may inhibit virus multiplication (BHOHWANI and RAZDAN, 1983).

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[1]The meristem is a dome of actively dividing cells, about 0.1 mm in diameter and 0.25 mm long, and is the centre of activity for various developmental programmes in the life of the higher plant.

To date, the only practical methods of eradicating viruses from vegetatively propagated species has been tissue culture, thermotherapy, or a combination of both (QUACK, 1977; WALKEY, 1980). In ornamentals, meristem culture is the most widely used procedure in commercial applications of tissue culture. As early as 1922, KOTTE and ROBBINS cited in KARTHA (1981), independently observed the growth of root tips on mineral solutions supplemented with sugars, aspargin, and peptin. Later, WHITE (1943) was able to subculture TMV-infected tomato roots *in vitro*. By dissecting such roots and testing the various zones by inoculation of a local lesion host of this virus, he noticed that the virus concentration in the terminal parts was low compared to that of basal parts . On the root tip he found no evidence of any virus at all. Likewise LIMASSET and CORNUET (1949) observed that in systematically infected plants, virus concentrations decreased as they approached the apical meristem, with no virus being detected in half of the cases. However, the history of meristem culture essentially began with the first successful culture of meristem tips of Nasturtium (*Tropaeolium majus*) and formation of rooted plants (BALL, 1946). This led MOREL and MARTIN (1952) to postulate that it might be possible to isolate the apical meristem of a systematically infected plant *in vitro* in order to obtain virus-free plants, genetically identical to the "mother plant". They succeeded in confirming this hypothesis by freeing the ornamental *Dahlia* from viruses (WALKEY, 1978). Later it became a routine operation for many ornamentals, especially cut flowers. Today, more then ever, growers are aware of the importance of the phytosanitary quality of propagation material and of the final product.

Besides virus elimination, the second most important field of application of meristem and shoot tip culture has been the rapid clonal multiplication of vegetatively propagated plant species (KARTHA *et al.*, 1977; KARTHA *et al.*, 1980; KARTHA, 1981; KARTHA *et al.*, 1981; GEORGE and SHERRINGTON, 1984; HUSSEY, 1986). The advantage in using axillary bud proliferation from meristem cultures as a means of regeneration, is that the method is less prone to the risk of genetic instability (MURASHIGE, 1977; GEORGE and SHERRINGTON, 1984) and the incipient shoot has already been differentiated *in vivo*.

Thus, to establish a complete plant, only elongation and differentiation are required. *In vitro* organogenesis and embryogenesis, on the other hand, must undergo developmental changes, which usually involve the formation of callus with subsequent reorganisation into plantlets. This has not been easy to achieve in most plants (HU and WANG, 1983).

Although the rate of plantlet multiplication by means of organogenesis and embryogenesis is astonishing, their regeneration capacity usually diminishes rapidly after a number of sub-cultures and eventually this morphogenic potential is completely lost (KEHR and SCHAEFFER, 1976; YIE and LIAW, 1977). The initial multiplication rate for axillary bud proliferation, on the other hand, is rather slow. The rate nevertheless increases during the first few subcultures and eventually reaches a steady plateau during subsequent subculture cycles (HU and WANG, 1983). The value of this method to the horticultural industry, is therefore immeasurable.

4.2 Materials and Methods

Although terminal cuttings with fully expanded leaves were chosen from random areas on the parent *Combretum bracteosum* shrub growing in the University Botanical Garden, the maturity of the tissue was chosen carefully. Initial experimentation indicated that young, actively growing tissue could not withstand the decontamination procedure, whereas the older woody material was very difficult to section and contamination was severe. Semi-hardwood explants were most suitable, however, they also had to be examined as some of the nodes did not develop axillary buds in the leaf axils, making their use impractical for experimental purposes. Cuttings were made in the early morning in order to avoid moisture stress.

Variability among replicates receiving the same treatment has frequently been commented upon (STREET, 1977). This was borne in mind during the preparation of plant material for experimentation. In each experiment, as many replicates were made as available plant material would allow.

Different explant types and the varying maturity of the explants used (explants taken during different times of the year) resulted in the requirement of different nutrients at different concentrations. It is for this reason that for both the liquid and solid media experiments both MS medium and WPM (Woody Plant Medium) were tested. The best gelling agent was also tested (involving Gelrite versus Agar), with superior results being obtained when using Gelrite. Gelrite was used in further experimentation.

A further complicating factor was the loss of a certain number of replicates in every treatment owing to fungal and bacterial contamination. The cultures were examined weekly and the contaminated or dead cultures were recorded and removed. Notes were made of the various explant responses to the respective treatments. Results were analyzed statistically using a general analysis of variance (ANNOVA).

4.2.1 Germination *in vitro*

Mature seeds were obtained from Kirstenbosch National Botanical Gardens (33°59′00″S 18°26′00″E). These mature seeds from the immediate past fruiting seasons (December 1997 - March 1998; December 1998 - March 1999; December 1999 - March 2000) were collected directly off the trees. The hard pericarp was removed and the excised embryos were hydrated in sterile distilled water over night. Once hydrated (Plate 8), the excised embryos' were sterilized in a 2% NaOCl solution (made up from Jik, a commercially available household bleach) for twenty minutes and rinsed three times with sterile distilled water. The entire embryo was placed into a culture bottle containing solid MURASHIGE and SKOOG (1962) nutrient media (MS) with no exogenously applied hormones. The embryos were incubated in complete darkness at 25°C, throughout germination and until the shoot comprised at least eight nodes.

These etiolated shoots were then divided into nodal explants and placed onto a solid MS medium containing 0.5 mg.ℓ^{-1} BA (this was later changed to Woody Plant Medium (LLOYD and McCOWN, 1980) to eliminate the risk of hyperhydricity). Prior to placing the nodes

onto the nutrient media, they were soaked in an ascorbic acid (500 mg.ℓ^{-1}) solution for two minutes in an attempt to reduce browning.

The nodal explants were incubated in an environment where cool white light tubes provided continuous light (PAR 67.7μmol.$m^{-2}s^{-1}$), and the temperature remained at 25°C for approximately six weeks or until the axillary bud(s) had elongated to a length of four centimeters or more. The elongating shoot was then excised from the node and again dipped in an ascorbic acid solution. Following the protocol referenced by CHAPULA (1981; 1983 a; 1983 b) in successfully initiating root development on excised shoots, the *Combretum bracteosum* shoot was placed onto rooting media (quarter strength Woody Plant Medium (WPM) containing 2g.ℓ^{-1} activated charcoal and 1 mg.ℓ^{-1} IBA) and incubated at 25°C in a 16: 8 hour light: dark cycle.

After root formation (an average of four weeks), the rooted explants were moved off the agar based media and into sterile vermiculite drenched with half strength Whites Basal Media (1934) *in vitro*. Plantlets were transferred into an *in vivo* environment after three weeks, where a potting media consisting of 2 parts sand: 1 part vermiculite and 1 part compost (volume: volume: volume) was used.

As micropropagated plants are generally susceptible to transplantation shock *Combretum bracteosum* plantlets had to be properly acclimatized when transferred from the *in vitro* environment to the soil. Potted plantlets were kept in the misthouse, which provided basal heating (18°C-21°C) and a high relative humidity (intermittent mist system). To reduce the severity of stresses that the acclimatizing plantlet was faced with, and to ensure that the mist spray did not leach available nutrients from the potting media, a half-strength MS medium solution (containing no sucrose) was applied as a soil drench every second week (on three consecutive occasions).

Depending on the season (mid-summer heat being detrimental), the plantlets were then

moved to the greenhouse, where the environmental conditions were more adverse, however, closer to their natural living conditions. Initially, the survival rate of the plantlets at this stage was unpredictable. Key factors that had a direct effect on plantlet survival were the fluctuating temperatures, a possibly unsuitable potting media composition and incorrect watering regime. As nothing could be done about the fluctuating ambient temperatures prevailing during periods of plantlet acclimation, the other two factors were addressed. Changing the potting media in the misthouse to perlite only, and retaining the original media for use in the greenhouse resulted in a more vigorous and voluminous root system. With regard to the watering frequency, plants were given a soil drench watering daily, in addition to the automated water sprinkling. Over time, the amount and frequency of water applied manually was decreased.

4.2.2 Axillary shoot elongation

For axillary shoot elongation, the most frequent explant type is a short, single-node stem section (HICKS and NAIR, 1986). Initially, *Combretum bracteosum* softwood and semi-hardwood shoots were placed into culture within hours of being removed from the parent plant. The efficiency of a single sterilant wash was however not sufficient as profuse fungal growth was evident after only a few days in culture.

KOWALSKI and VAN STADEN (1998) suggested that supplementing the decontamination protocol with a cold pre-treatment reduced explant loss. The explants (Plate 8) therefore, once cut from the parent plant had their leaves removed at the petiole and were stored in the fridge (±5°C) for 5 - 7 days. Following that, the *C. bracteosum* "shoots" were soaked in Benlate® for thirty minutes and then placed into a solution containing 500 $mg.\ell^{-1}$ ascorbic acid and 250 $mg.\ell^{-1}$ citric acid for ten minutes. This reduced the production of phenolics by the stressed explants. The "shoots" subsequent to being dipped in 70% ethanol for twenty seconds, were either soaked in 2% NaOCl or 0.1% mecuric chloride ($HgCl_2$) for twenty or fifteen minutes respectively. Different sterilants were used according to the season during which the explants were collected. Spring and early summer growth did not require a strong

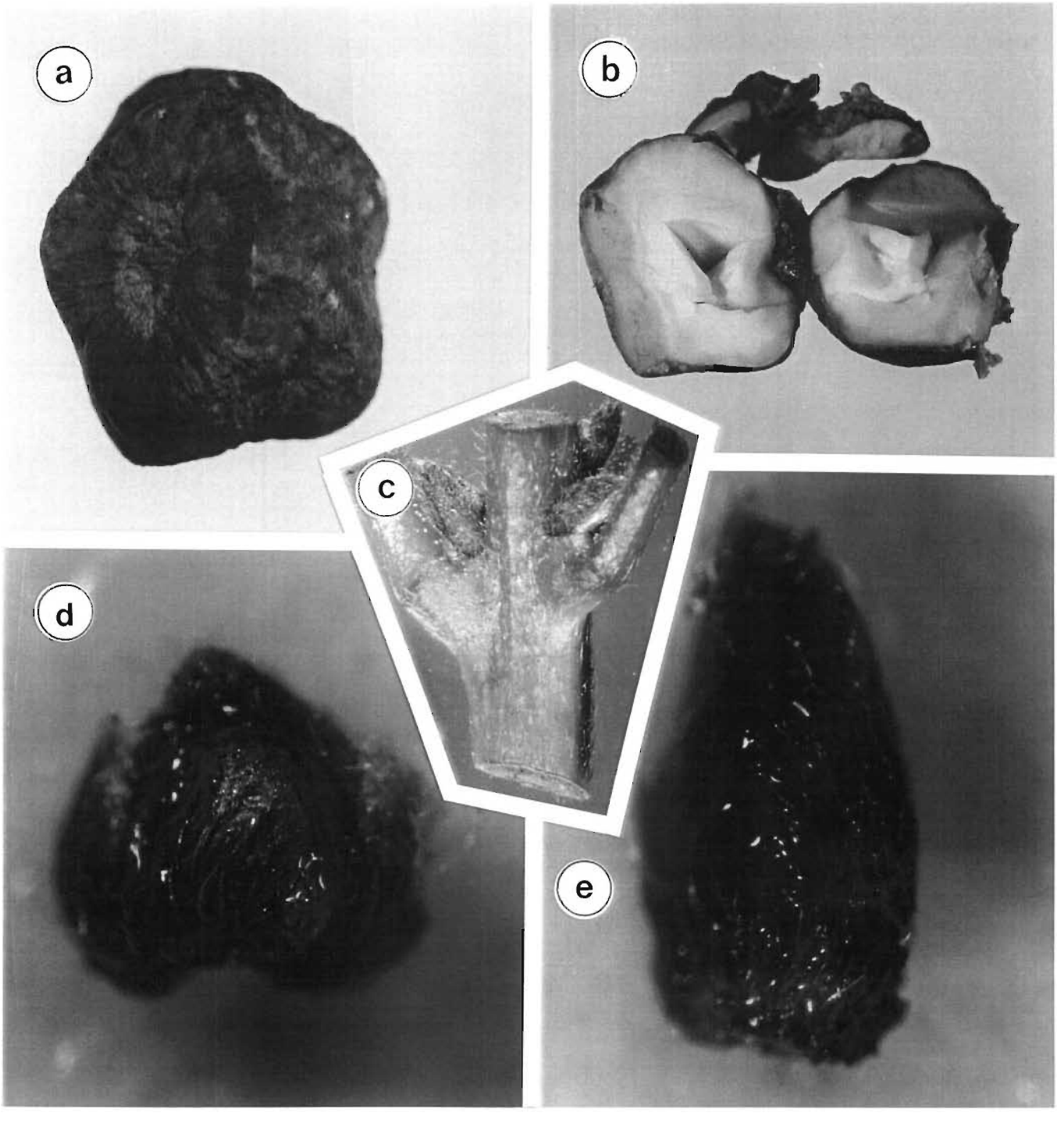


Plate 8: Appearance of *Combretum bracteosum* explants used *in vitro* (a) and (b) hydrated seed (± 20 mm); (c) single node (± 10 mm); (d) axillary meristem (± 0.9 mm); (e) apical meristem (± 1 mm)

sterilant, with the household bleach, NaOCl (2% for 20 minutes), proving adequate. Explants taken from growth in late summer and autumn were prone to high contamination levels and required a stronger sterilant (0.1% mecuric chloride, for 15 minutes). Although the semi-hardwood explants proved mature enough to withstand the mecuric chloride soak, the poor success rate in initiating shoot elongation so late in the growing season (February - September) did not warrant further experimentation. Tween-20 was used as a surfactant during decontamination (1 - 2 drops Tween-20 per 100 ml solution). Three ten minute washes in sterile distilled water was sufficient to remove the sterilant and surfactant from the explant tissue. On completion of surface sterilization, the plant material was sectioned into single node explants and placed into culture tubes containing a MURASHIGE and SKOOG (1962) or Woody Plant Medium for incubation.

It is documented that temperature affects all physiological processes (largely mediated by it's effect in chemical reactions) and is an important variable influencing the development of explants (WENT, 1953). It was important therefore to determine and retain incubation temperatures at their optimum for healthy *Combretum bracteosum* development *in vitro*. Explants were incubated in a growth chamber where the temperature was maintained at 23°C - 27°C, the total irradiance was approximately 67.7 $\mu mol.m^{-2}s^{-1}$ and the cool white light tubes operated on a 16-hour light, 8-hour dark cycle.

In order to decrease the browning associated with stressed explants, numerous different techniques were tested in order to improve the survival rate of the excised nodal explants. A pre-treatment of soaking explants in a solution of 500 $mg.\ell^{-1}$ ascorbic acid and 250 $mg.\ell^{-1}$ citric acid was tested initially. As an alternative, the application of UniLABs' water-soluble PVP (molecular weight 700,000) to the nutrient media at levels of one, two or three grams per liter, and/or 75 $mg.\ell^{-1}$ ascorbic acid was also considered. The final alternative was to add one, two or three grams activated charcoal to the media.

Different hormone concentrations and combinations were tested in an attempt to obtain the

healthiest and most vigorously growing plantlets ultimately available for commercial distribution. An attempt was made to initiate the elongation of the axillary bud which appeared to be dormant. To do this, cytokinins and auxins were applied to the nutrient media, both alone and in combination.

Hormone grids consisting of the combinations BA: IAA; KIN: IAA; KIN: NAA; BA: NAA proved most successful and stable (when the experiment was repeated, resulting trends and morphological developments were always the same. Other hormones (and combinations thereof) tested produced results that differed considerably with each repetition carried out - and no results, that could be used successfully for further analysis, could be achieved). Using the above mentioned hormone grids to induce axillary bud break, the combination of the auxin NAA and the cytokinin BA, proved most effective. As the concentration level of 1.0 mg.ℓ^{-1} BA: 0.5 or 1.0 mg.ℓ^{-1} NAA appeared to produce optimal results, these hormone concentrations were used in further experimentation. Slow progress with this hormone combination necessitated further experimentation for alternative options. Previous success with Kelpak motivated the decision to test it again, this time however, in a sterile environment. The seaweed concentrate (SWC) was added to the Woody Plant Media prior to autoclaving at 0.25, 0.5 and 1.0% of the standard commercially available Kelpak solution.

Once the elongating shoot had developed at least four nodes, it was excised from the nodal explant, dipped into a solution containing ascorbic acid and then placed onto a rooting medium. The medium which brought about best results at this stage was a quarter strength Woody Plant Medium, containing 1.0 mg.ℓ^{-1} IBA. As a high light intensity has a negative or inhibitory effect on root development and growth, charcoal (2 g.ℓ^{-1}) was added to the medium during this culturing stage to ensure reduced light levels at the site of root initiation.

Once the roots had developed on the excised shoot, the same protocol as outlined in Chapter 4.2.1 was followed.

4.2.3 Meristem Culture

Meristems should preferably be taken from actively growing buds (GUPTA *et al.*, 1981). The explants taken from the tip of the cutting were in a more juvenile stage of development than those taken from the base. Due to the presumably stronger growth potential of the younger terminal buds over the lateral ones, the former are often better for shoot regeneration (VERTESY, 1979). However, due to limited plant material, both apical (Plate 8 d) and the older axillary (Plate 8 c) meristematic regions were used for experimental purposes.

As with other types of tissue culture, an essential step in raising meristem-tip cultures is to obtain explants free of surface pathogens. Usually the meristem tips are so well protected with overlapping leaf primordia, that a surface decontamination is not essential. However, as a precaution, the cuttings were dipped in ethanol for 20 seconds and then soaked in 2% NaOCl for 20 minutes. After being rinsed with distilled water thrice, the axillary buds and apical meristems were excised from the cutting, soaked in an ascorbic and citric acid mixture (500 $mg.\ell^{-1}$ and 250 $mg.\ell^{-1}$ respectively) and placed onto the culture medium.

Meristems cultured during spring and early summer were able to overcome the stress of being removed from the parent plant far better than meristems cultured during late summer and autumn. However, once established on the nutrient media, the latter displayed growth as vigorous as spring cultured explants, with the early summer initiated explants developing at a slower rate. Although the vigor of explants varied, their response to exogenous hormones enabled meristem culture experiments to be carried out for eight months of the year (September -April).

With the aid of a dissecting microscope, the outer leaf primordia surrounding the meristematic area were removed. Experimentation showed that although the chance of viral contamination was increased with the larger explant size (QUACK, 1977), explants consisting of the meristematic zone, together with three to five young leaf primordia

(approximately 0.5 - 1.2 mm long) were optimal for culture, as their survival rates were much higher. Care had to be taken to prevent the dessication of the meristems due to the constant flow of air through the laminar flow bench and the heat generated by the dissecting microscope. The excised meristems (Plate 8 d and e) were placed onto the nutrient media and incubated. Being so small, it was difficult to ensure that the explants were placed onto the solid nutrient media with the correct orientation. WANG and HU (1980) however, indicated that the orientation of the meristem on the medium does not seem to be critical. This also proved to be so for *Combretum bracteosum* explants.

Both liquid and solid media were used in experimentation. The first liquid media experiment consisted of explants being fully submerged in the media (five flasks, with ten explants per flask for each media type, MS or WPM). Although aeration was provided by placing the flasks on a rotary shaker, immersing the meristems in the liquid media proved unsuccessful as the explants became brown within hours of submergence.

The other liquid medium experiment consisted of the meristem explants being suspended above the liquid media (MS or WPM) by means of a filter paper bridge. Diffusion of medium across the filter paper enabled the meristem to be fed continually by the nutrients and hormones, yet eliminating loss of explants due to insufficient aeration. The early filter paper bridge experiments, although unable to stimulate morphological development without the aid of exogenous hormones, were successful in that the explants remained alive for three weeks or more. Further experimentation showed that the addition of exogenous hormones to the nutrient media stimulated morphogenetic responses from the meristems. These experiments consisted of thirty tubes per treatment, where the hormone grid comprised a combination of BA and IAA or NAA (concentrations 0 $mg.\ell^{-1}$, 0.1 $mg.\ell^{-1}$, 0.5 $mg.\ell^{-1}$ and 1.0 $mg.\ell^{-1}$ for each hormone).

The application of exogenous hormones stimulated the increase in meristem volume, the opening of the excised axillary or apical bud scales, and in some cases initiated bud scale

elongation. However, this is where the development ceased and there was never evidence of shoot development or elongation. The application of GA_3 to the media, intended to stimulate the elongation of the developing shoot from the central meristematic zone. Concentrations of 0.1 mg.ℓ^{-1}, 0.5 mg.ℓ^{-1} and 1.0 mg.ℓ^{-1} were tested.

After five weeks on the liquid media containing the auxins, cytokinins and gibberellin, the explants' well-being appeared to be deteriorating. They were then sub-cultured onto a solid hormone-free media containing charcoal in order to rid the explant of excess hormones that may stunt or inhibit development by becoming toxic. The charcoal (tested at 1 g.ℓ^{-1}, 2 g.ℓ^{-1} and 3 g.ℓ^{-1}) however, did not provide a solution as all the explants placed onto the media (both MS and WPM) died within a week.

The many variables associated with the filter-paper bridge method, became problematic. After a few weeks of saturation, the filter paper became flimsy and started sagging. The suspended meristem as a result was squashed (the opening bud scales were broken off) and the light was blocked from reaching the meristem. Although some positive results were obtained using the liquid media and filter paper bridges, placing meristem explants directly onto solid nutrient media proved more successful in the long run. This however was also problematic as the surface in direct contact with the media became white and disfigured. The solution to this was to place a Whatman No. 2 filter paper disc on the media surface as it was able to act as a buffer for the sensitive meristem. The greater ease of handling the petri-dishes also added to its preference in attempting to establish plantlets generated from meristematic explants.

The climatic zone where the plant originated from offers some clues as to the optimum physical incubation conditions for an explant (ZIV, 1979; BHOJWANI, 1980). Theoretically therefore, a constant incubation temperature ranging between 24-26°C and a high light intensity was considered suitable to induce healthy *Combretum bracteosum* plantlet formation. Incubation temperatures of the explants were maintained between 23°C

and 27°C although the light regimes varied from a 24 hour dark cycle to a 24 hour light cycle, with intermediates (10:14 hour light: dark and 16: 8 hour light: dark cycle) also being tested. The best results were obtained from meristems incubated in the 16: 8 hour light: dark cycle. This light regime was used in further experimentation.

An alternative to the standard exogenous hormones applied in tissue culture was also used, with satisfying results. Kelpak, the brown algal extract which contains cytokinins and auxins (CROUCH and VAN STADEN, 1990), was added to the solid nutrient media at 0.25%, 0.5% and 1.0% of the concentrated solution. Five meristem explants were placed on to the filter paper disk, with ten petri-dishes per treatment. These explants were incubated in the same conditions specified above. Once these explants had responded to the Kelpak, they were sub-cultured onto a hormone-free media (MS and WPM, with and without charcoal) as well as onto media containing GA_3 (0.1; 0.5 and 1.0 mg.ℓ^{-1}). This was an attempt to stimulate further meristematic activity from the Kelpak induced shoot initial.

4.3 Results and Discussion

An important aspect in the micropropagation of plants is explant choice. In many cases only a few tissues will give the desired response. Tissues in some parts of the shrub display delayed maturation when compared to other regions on the same plant. Of the numerous *Combretum bracteosum* explant types tested, positive results obtained (ie. hardened off plantlets being generated by end of tissue culture protocol) for further commercial use were from the seed (for *in vitro* germination) and nodal explants (for axillary shoot elongation).

Another critical factor in the success of *Combretum bracteosum* micropropagation was the fact that there are specific periods during the annual growth cycle when some tissues and meristems display an increased morphogenetic plasticity. As recommended by BONGA and VON ADERKAS (1992), it was important to excise the right tissue at the right time for use as explants. *Combretum bracteosum* explants displayed greatest plasticity during the spring months, with the seasonal variation rendering *in vitro* explants least responsive during late

autumn and winter.

Seasonal variation was also evident with explant browning intensity. During spring months phenolic production in newly excised explants was most problematic. Browning is believed to be a shock response to excision, as it appeared within hours of plant material being placed in culture. The release of these phenolics into the agar not only inhibited development, but lead to the premature death of the explants. CHALUPA (1987) suggested four methods that could suppress browning. These are:

1. Transplanting explants onto fresh medium whenever browning occurred;
2. Soaking newly excised explants in a sterile solution of antioxidants, such as ascorbic and citric acid;
3. Growing explants on a nutrient media containing antioxidants (ascorbic acid, polyvinylpolypyrrolidone) or charcoal; and
4. Incubating explants in low light intensities or darkness.

The first of the above mentioned options was not practical. The rapid rate at which the phenolic leachate penetrated the basal medium rendered this option too time consuming and expensive. The second option was the most effective and used in all further experimentation. Treated with an ascorbic and citric acid dip, the explants showed no sign of browning. In addition there was no detrimental effects caused by the anti-oxidants. During the spring months more young nodal explants were able to overcome the stress of excision by placing some ascorbic acid into the media, thus reducing the amount of phenolics produced (this was not necessary for meristem explants).

Nodal explant growth and survival was reduced by the presence of PVP and activated charcoal in the nutrient media. This negative effect caused by the charcoal could be attributed to the fact that besides adsorbing the phenolics produced by the explant, ingredients essential to proper growth had become unavailable as they were now bound to the activated charcoal as well. Mineral analysis of the medium after treatment by activated

charcoal indicated a specific absorption of Fe and Zn (MISSION *et al.*,1983) cited in GASPAR and COUMANS (1987).

Combretum bracteosum explants also proved sensitive to basal media type. *In vitro* responses to the different media types are highly species specific and vary from little difference in growth between media to a life-or-death reaction for others. It was suggested that in initiating a new woody plant species in culture, first to use a basal media with a low salt content (eg. WPM), and then later determine whether a higher nutrient content provides optimal growth. Unlike the high salt media (eg. MS), the low salt media are seldom so inhibitory as to be lethal, although they may not be optimal (McCOWN and SELLMER, 1987). The plethora of media formulations for the culture of woody plants which currently appear in the literature are as a result of early attempts to culture shoots from woody species. It soon became apparent to tissue culturalists that the standard media formulations commonly used for herbaceous plants (eg. MURASHIGE and SKOOG, MS) did not support good growth of many woody plants (McCOWN and SELLMER, 1987) and more specific formulations had to be sought.

In carrying out axillary shoot elongation and the meristem culture experiments on *Combretum bracteosum*, both MS media and WPM were used. The compositional difference of these two media types is attributed to the total ionic strength of the formulations, in that the WPM comprises a lower salt content when compared to the MS media. The nutrients contributing in a major way to the ionic strength of a medium are the macro-elements, in particular, the nitrogen and potassium sources. Thus, any major reduction of the ionic strength of a medium must involve reductions in the levels of these nutrients.

In comparing developmental responses of *Combretum bracteosum* explants on the two basal media types, there was initially no difference. It was only after six to eight weeks of incubation on MS media that the newly developed shoots and foliage (on the nodal explants)

were characterized by a watery, translucent and often swollen appearance - indicating hyperhydricity. This phenomena, however, appeared to be random as within the same treatment, it was possible to see both hyperhydric and normal leaf clusters. Foliage of the non-hyperhydric explants appeared flat and well developed while the hyperhydric ones were either a shiny dark or a dull green. They sometimes showed a mosaic of dark green and dull green spots. CHALUPA (1987) states that finding hyperhydric plants among healthy explants is not unusual, with more extreme cases even exhibiting axillary buds from one plant developing hyperhydric and normal shoots at the same time.

Hyperhydricity, a multi-faceted phenomenon, is a physiological disorder frequently affecting herbaceous and woody plants during *in vitro* vegetative propagation (GASPAR *et al.*, 1987 b). DEBERGH *et al.* (1981) stated that the source and the physiological condition of the mother plant at the time of taking cuttings and inoculating the excised explants do not interfere with hyperhydricity. All the suggested causes refer exclusively to *in vitro* conditions. Here it is interesting to note that most reported cases of hyperhydricity have occurred on the mineral medium of MURASHIGE and SKOOG (1962). The effect of the ammonium rich culture media may be due to the ease of ammonium ion absorption, inducing a fall in the C/N ratio and consequently a decline in the synthesis of cellulose and lignin. This decline might be responsible for the glassy turgescence of the tissues via reduced wall pressure in turn increasing water availability (DEBERGH, 1983).

Cell hyperhydricity may also be influenced by agar concentration, as by decreasing the agar concentration the water potential changes, in turn causing the water availability to increase (DEBERGH, 1983; BORNMAN and VOGELMANN, 1984; VON ARNOLD and ERICKSONN, 1984; KEVERS and GASPAR, 1985; VIEITEZ *et al.*, 1985). Connected to the high matrix potential (low agar concentration) is cytokinin availability - the increased ease in absorption of this hormone therefore cannot be ruled out as a cause of hyperhydricity. Hyperhydricity is sometimes reversible (VON ARNOLD and ERICKSONN, 1984). Transferring the *Combretum bracteosum* explants onto a non-

hyperhydricity-inducing medium enabled new shoots and leaves to develop normally. Increasing the agar concentration within the media proved insufficient, however further experimentation showed that changing solidifying agents from agar to Gelrite eliminated the problem of hyperhydricity. Gelrite is likely to be more efficient in maintaining lower water vapour levels in the culture environment (AITKEN-CHRISTIE and JONES, 1985; GASPAR *et al.*, 1987), as well as being a better binding substrate for the growth regulators (slower absorption by explant), and chloride and ammonium ions (DEBERGH, 1983).

The failure of HABERLANDT's (1902) attempts to obtain cell divisions in isolated cells placed on nutrient media was attributed to the lack of certain "substances that control cell division". Following the discovery of auxin by WENT (1926) and its chemical identification by KÖGL *et al.*, (1934) several researchers simultaneously demonstrated the usefulness of this hormone in cell cultures (WHITE, 1939). Variations in the sensitivity of different tissues to IAA and other synthetic auxins were soon discovered (FOX, 1963; WOOD *et al.*, 1969; DYSON and HALL, 1972).

Another milestone in establishing the importance of growth regulators in plant cell cultures came in 1955 with the discovery of cytokinins as regulators of cell division (MILLER *et al.*, 1955 a; MILLER *et al.*, 1995 b). This was soon followed by a discovery with far reaching implications - that the relative concentrations of an auxin and a cytokinin controlled the morphogenetic response of tobacco cell tissue in culture (SKOOG and MILLER, 1957). Although this observation was confirmed in several tissues it soon became apparent, however, that morphogenesis in cell cultures was controlled by plant growth regulators in a variety of ways (STUART and STREET, 1971; STREET, 1977; KOHLENBACH, 1978).

Each type of plant growth regulator has a wide range of physiological effects in different plants. These effects are determined by the kind of growth regulator, its concentration, the presence or absence of other growth regulators, and by the genetic makeup and the physiological status of the target tissue. The same physiological response in different

tissues, even of the same plant, may require different growth regulators or different combinations of growth regulators. Synergism and quantitative interaction of two or more growth regulators are a common occurrence (MINOCHA, 1987). A growth regulator that elicits a positive response in a given tissue at a given concentration may inhibit the same physiological response when used at higher concentrations.

4.3.1 *In vitro* germination

Once overcoming contamination problems, *Combretum bracteosum* seeds were easy to germinate. The vigorous growth of the etiolated plantlets enabled a rapid rate of plant regeneration, which is promising as it would be ideal to the commercial propagator. Each node sub-cultured onto media containing 0.5 mg.ℓ^{-1} BA (Figure 11) was able to generate two shoots (one from each axillary bud). Once that shoot had elongated and had been excised, the same node was able to generate another shoot or two from the same source - this was remarkable as nodal explants (discussed in Chapter 4.3.2) did not display this phenomenon. Lower concentrations of BA (0.1 mg.ℓ^{-1}) were not as effective in stimulating morphological changes to the axillary bud, with only a slight swelling evident. BA concentrations of 1.0 mg.ℓ^{-1} lead to callus development on all the cut surfaces as well as on the axillary buds themselves.

Root induction on the shoots was not difficult (Figure 12). The vigor and volume of the root systems in culture were not as substantial as desired (Plate 9). However, within a few weeks of acclimation considerable increases were observed in both vigor and volume. This was thought to be a consequence of the plantlets having to absorb nutrients and water which are no longer readily available to them, as well as having to anchor itself into the potting medium.

The factor proving most detrimental to plants during the acclimation phase was the fluctuating ambient temperatures. The stress imposed on the plantlets however, in most instances could be avoided. Bearing in mind that the micropropagules lacked sufficient

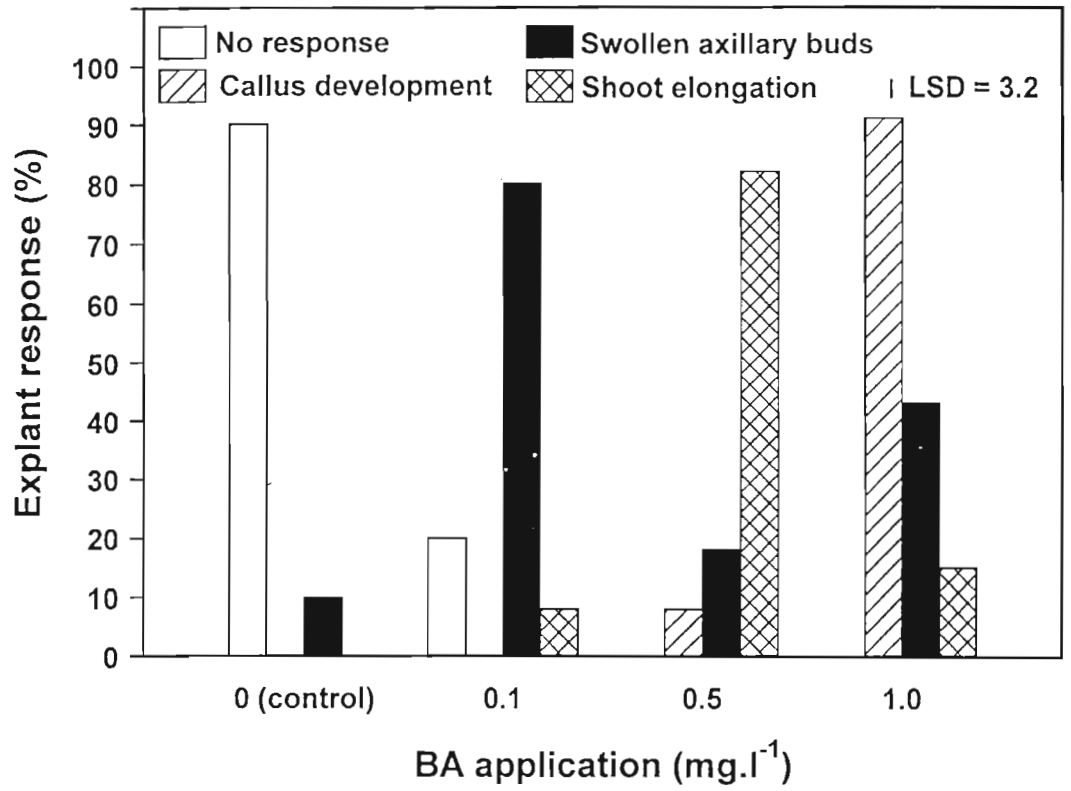


Figure 11: Shoot elongation from explants originating from *in vitro* germinated *Combretum bracteosum* seed, stimulated by different levels of BA

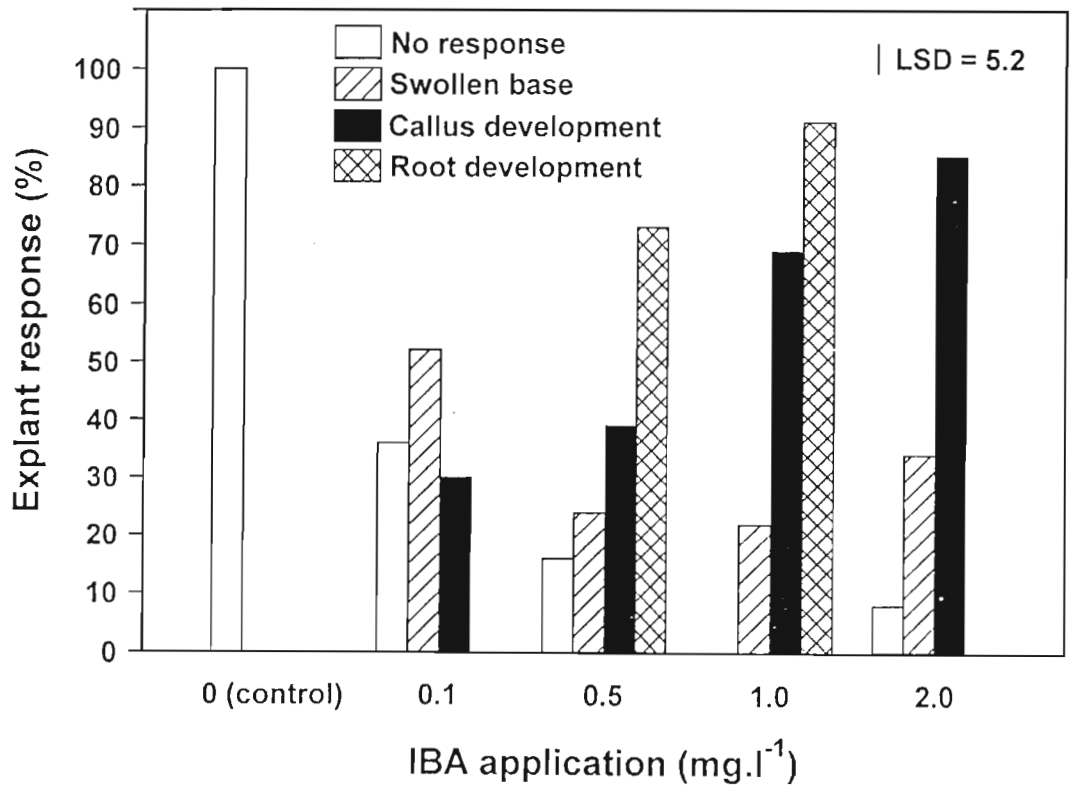


Figure 12: *In vitro* root induction on *Combretum bracteosum* shoots

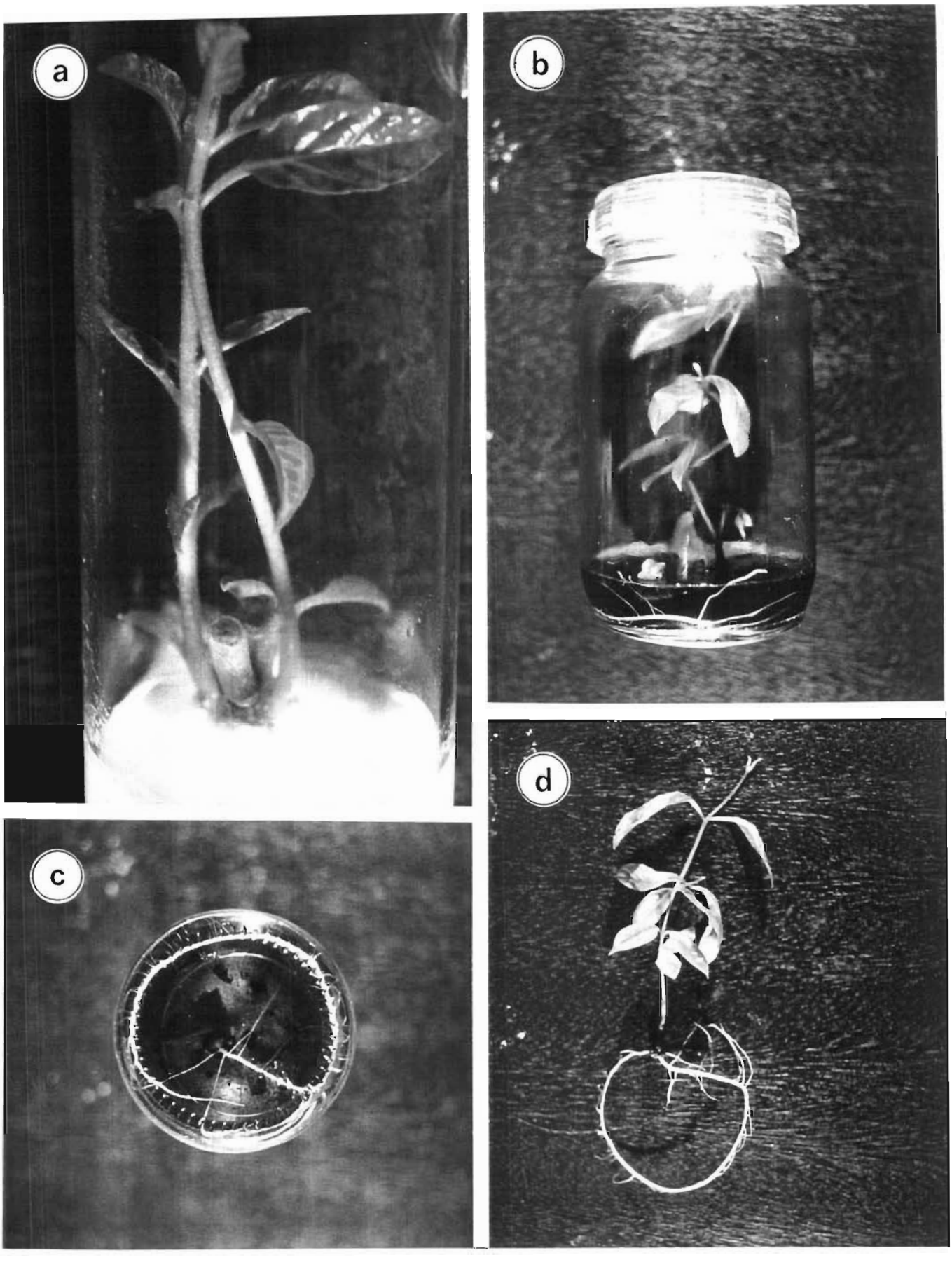


Plate 9: Developmental stages of *Combretum bracteosum* plantlet subsequent to shoot elongation (a) shoot elongation from nodal explant; (b) sub-cultured shoot on rooting medium (IBA); (c) morphology of *in vitro* developed roots; (d) plantlet ready for acclimation in misthouse

epicuticular wax and had abnormal stomata, the high atmospheric humidity in the misthouse ensured that no excessive dehydration and poor control of gas exchange resulted. During "heat-wave" periods, extending the plantlets' incubation time in the misthouse aided in their survival. As vegetative development on the plantlets progressed and the shoots developed new, functional leaves, the temporary hindrance posed by the lack of proper stomata and epicuticular waxes was overcome. Having undergone these modifications, plantlets were able to withstand high ambient temperatures.

Care also had to be taken in encouraging plantlets to produce their own "food". In having provided all their required nutrients previously in the culture media, *in vitro* generated leaves often have an inadequate photosynthetic apparatus (WETZSTEIN and SOMMER, 1982; SMITH *et al.*, 1986; SWARTZ and LINDSTROM, 1986; DHAWAN and BHOJWANI, 1987; HUTCHINSON and ZIMMERMAN, 1987). The sucrose in the nutrient medium is partly responsible for this as it suppresses the formation of ribulose biphosphate carboxylase, a key enzyme in photosynthesis (FLECK *et al.*, 1982). Therefore, starting with rooted plantlets growing within the vermiculite/ liquid Whites nutrient media combination, careful care was taken to acclimatize the plantlet not only to extrinsic factors, but to equally important intrinsic factors. In reducing the available sucrose gradually the photosynthetic mechanism of the plant was able to recover and support the plantlet independently.

4.3.2 Axillary shoot elongation

The discovery of the morphogenetic effects caused by the interaction of auxin and cytokinins, has influenced tissue culturalists to the extent that it is usually the first set of treatments given to cell cultures of any new tissue culture specimens. However, the question as to how the exogenous concentrations of growth regulators affect the endogenous levels of these substances at the critical time of the induction of a morphogenetic response has not been answered. *Combretum bracteosum* nodal explants responded to the hormone combination of BA and NAA (Figure 13) by producing an elongated shoot from one of the three axillary buds at the node. The slow growth response to this hormone combination was

not ideal, as in a commercial set-up long waiting periods between sub-culturing and the amount of growth-room space taken up by the explants would limit this ornamental's popularity. Cytokinin concentrations (1.0 mg.ℓ^{-1} BA) combined with an auxin (NAA) level of 1.0 mg.ℓ^{-1} or 0.5 mg.ℓ^{-1} stimulated axillary shoot elongation after five weeks (Plate 10 e). After being placed on the culture media, the explants would remain static for the first two weeks, after which swelling of the bud became apparent. A developmental trend was apparent, as increasing cytokinin levels induced more prolific axillary bud morphological changes and subsequent shoot elongation (Figure 13 and Plate 10). Unfortunately, the rate

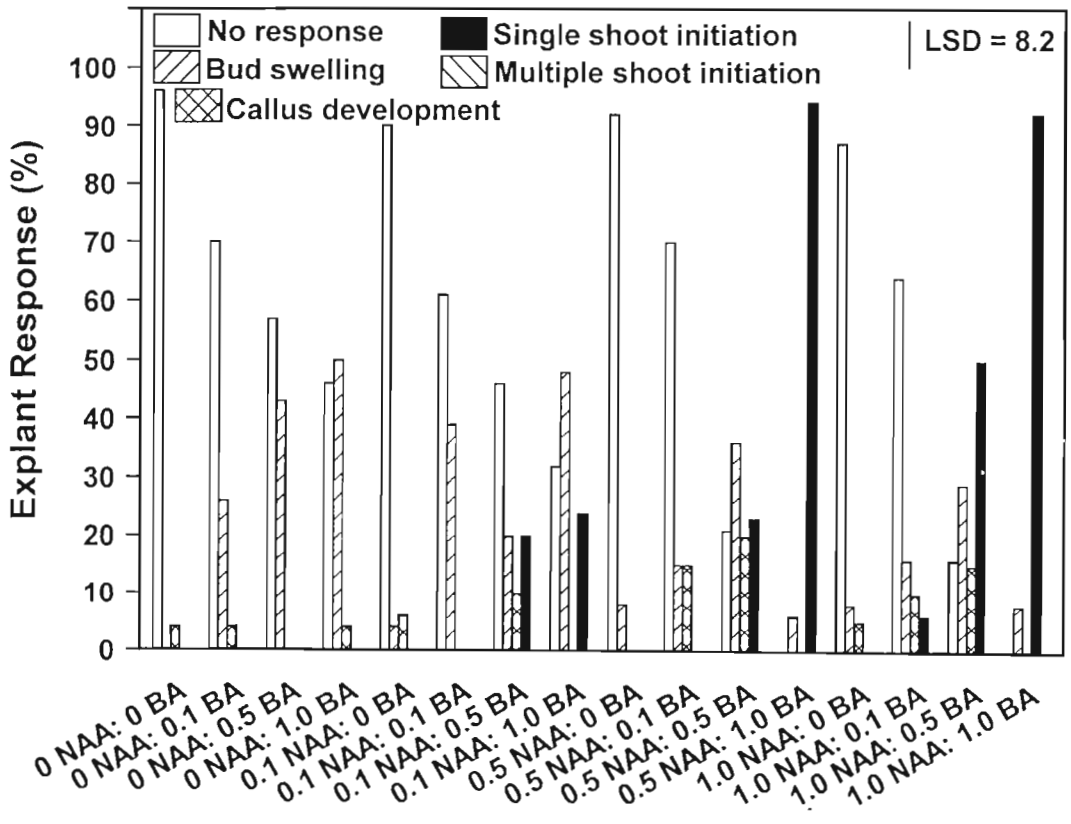


Figure 13: Initiation of axillary shoot elongation in *Combretum bracteosum* nodal explants using different combinations of cytokinins and auxins

Plate 10: Typical swelling of axillary buds or shoot elongation on *Combretum bracteosum* nodal explants induced by an auxin: cytokinin combination

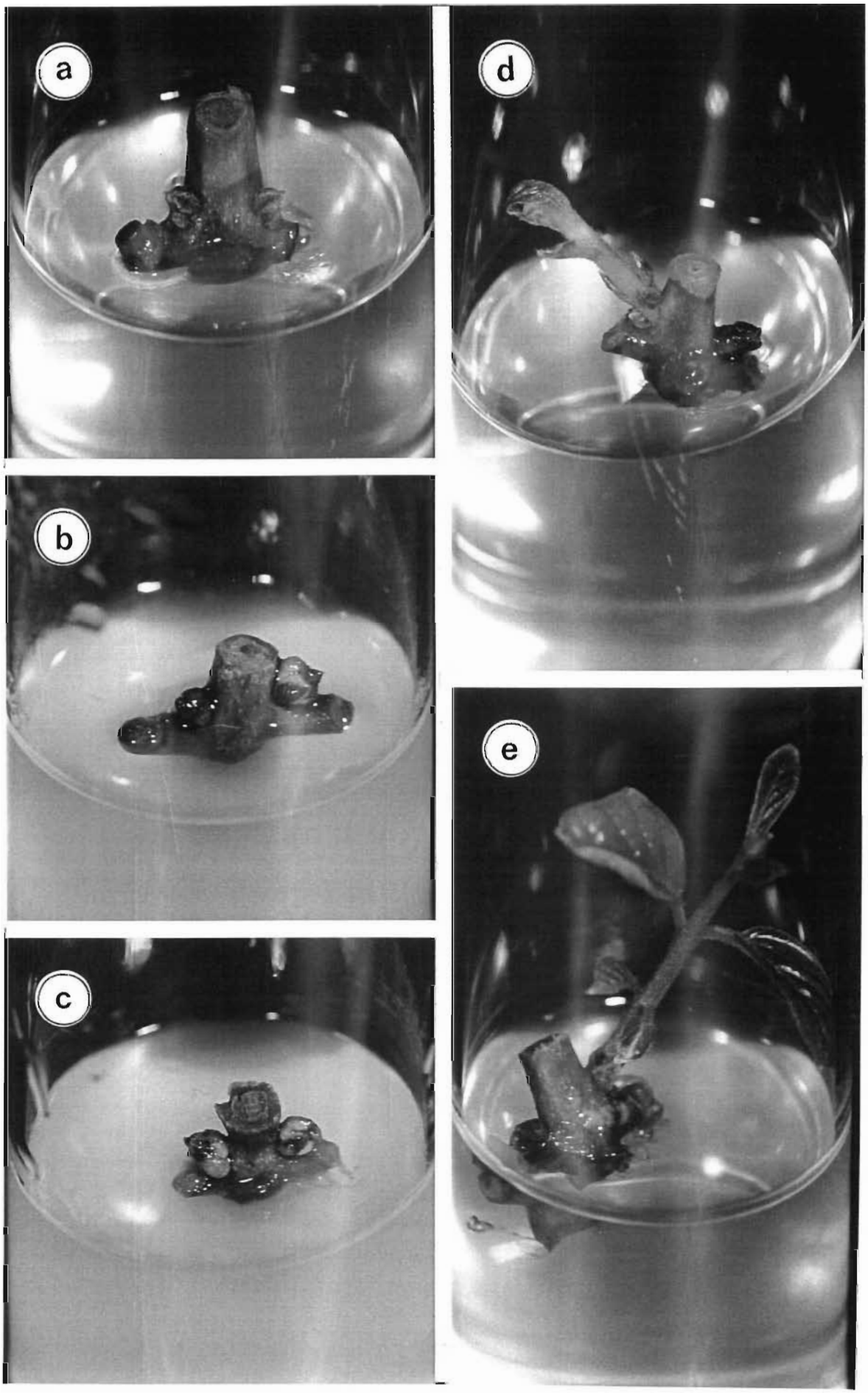
(a) No morphological changes to explant on control media (no hormones);

(b) single swollen axillary bud (0 mg.ℓ^{-1} NAA: 0.1 mg.ℓ^{-1} BA);

(c) two swollen axillary buds (0.1mg.ℓ^{-1} NAA: 0.1 mg.ℓ^{-1} BA);

(d) single shoot elongation (0.5 mg.ℓ^{-1} NAA:1.0 mg.ℓ^{-1} BA); and

(e) single shoot elongation (1.0 mg.ℓ^{-1} NAA: 1.0 mg.ℓ^{-1} BA).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at which these changes took place was slow. It is documented, that the addition of a cytokinin to the basal media can induce and promote multiple shoot development (MINOCHA, 1987). *C. bracteosum* has the potential to produce three shoots per explant (or as many as six on multiple shoot stimulation). Using BA and NAA however, only one shoot developed. Although this was a positive result, ignoring the potential of a further two shoots elongating was impossible (Plate 10 d and e; Plate 11 b, c and d).

Besides experimentation carried out by FINNIE and VAN STADEN (1985), DE WAELE *et al.*, (1988) and CROUCH (1990), there are few records of Kelpak utilization in *in vitro* plant propagation systems. The positive results gained in inducing roots on the difficult-to-root *Combretum bracteosum* cuttings, suggested the possibility that these products may prove useful in this *in vitro* propagation system. CROUCH (1990) showed when working with potato (*Solanum tuberosum* L. cv. van der Plank) that the seaweed concentrate had the ability to enhance the growth of the *in vitro* plantlets as well as *in vivo* plants. As the media used in the study contained all the essential nutrients required for normal plant growth, it is unlikely that the effect of SWC was due to mineral elements alone. The marked physiological responses of nodal explants to seaweed treatment may be due to plant growth regulators. PGR's are known to effect apical dominance. Identification of these compounds in commercial seaweed preparations is well documented. Auxins, gibberellins, cytokinins, and ABA (KINGMAN and MOORE, 1982) are known to occur in seaweeds.

The explant responded rapidly to applied SWC (Figure 14). The two week stress-induced delay period (displayed by the BA: NAA treated explants after excision) was eliminated, with the buds swelling and bud scales opening within days of being placed onto the media. The explant itself also remained in a healthy state for longer than previously. However, the high Kelpak concentration (1.0%) tended to cause the base of the explant (where in contact with the agar) to become white and develop a warty external appearance, to induce callus development on the top and base of the explant, as well as over the axillary buds (Plate 11 e - g). Although multiple shoot elongation was induced by both 0.25% and 0.5% Kelpak

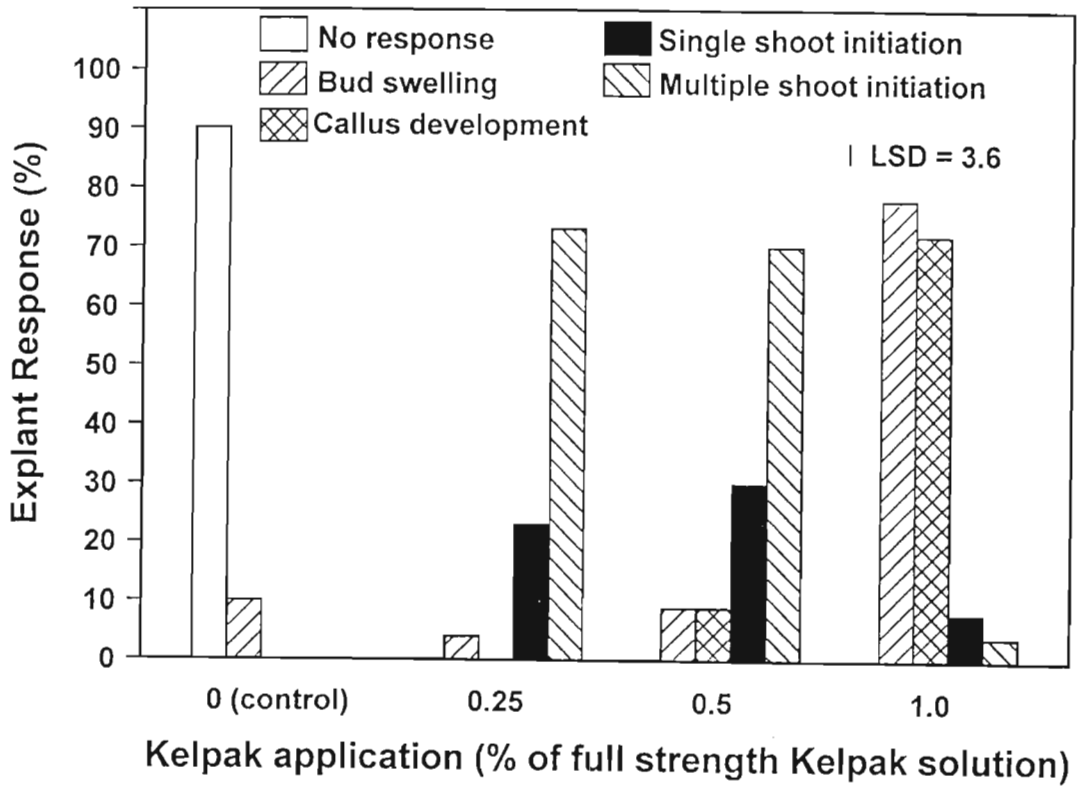


Figure 14: Initiation of axillary shoot elongation in *Combretum bracteosum* nodal explants using Kelpak

concentrations, shoots developed from treatment with 0.25% responded more favourably to subsequent exogenous hormone applications. The shoots (± four centimetres) were ready for excision after approximately four weeks. The excised shoots, having been placed onto a 1.0 mg.ℓ^{-1} IBA and 2 g.ℓ^{-1} charcoal containing quarter-strength WPM were able to produce roots more readily than shoots emanating from the media comprising BA: NAA.

The initiation of root formation is a complex morphogenetic phenomenon, in which extrinsic (activated charcoal, exogenous PGR's (GASPAR and COUMANS, 1987)) and intrinsic factors (temperature, light intensity, oxygenation, nutrients available (DRIVER and SUTTLE, 1987; HORGAN and HOLLAND, 1989)) play a role. Since the developing young shoots are a rich source of auxin production, the addition of exogenous auxin to rooting media becomes unnecessary in many species (LEE *et al.*, 1977; MEREDITH, 1979; HASEGAWA, 1980; PAPACHATZI *et al.*, 1981; MINOCHA, 1987). *Combretum*

Plate 11: *In vitro* shoot development and elongation of *Combretum bracteosum* nodal explants, stimulated by Kelpak

(a) No morphological changes to axillary buds on control media (no hormones);

(b) elongation of two shoots from axillary buds (0.25% Kelpak);

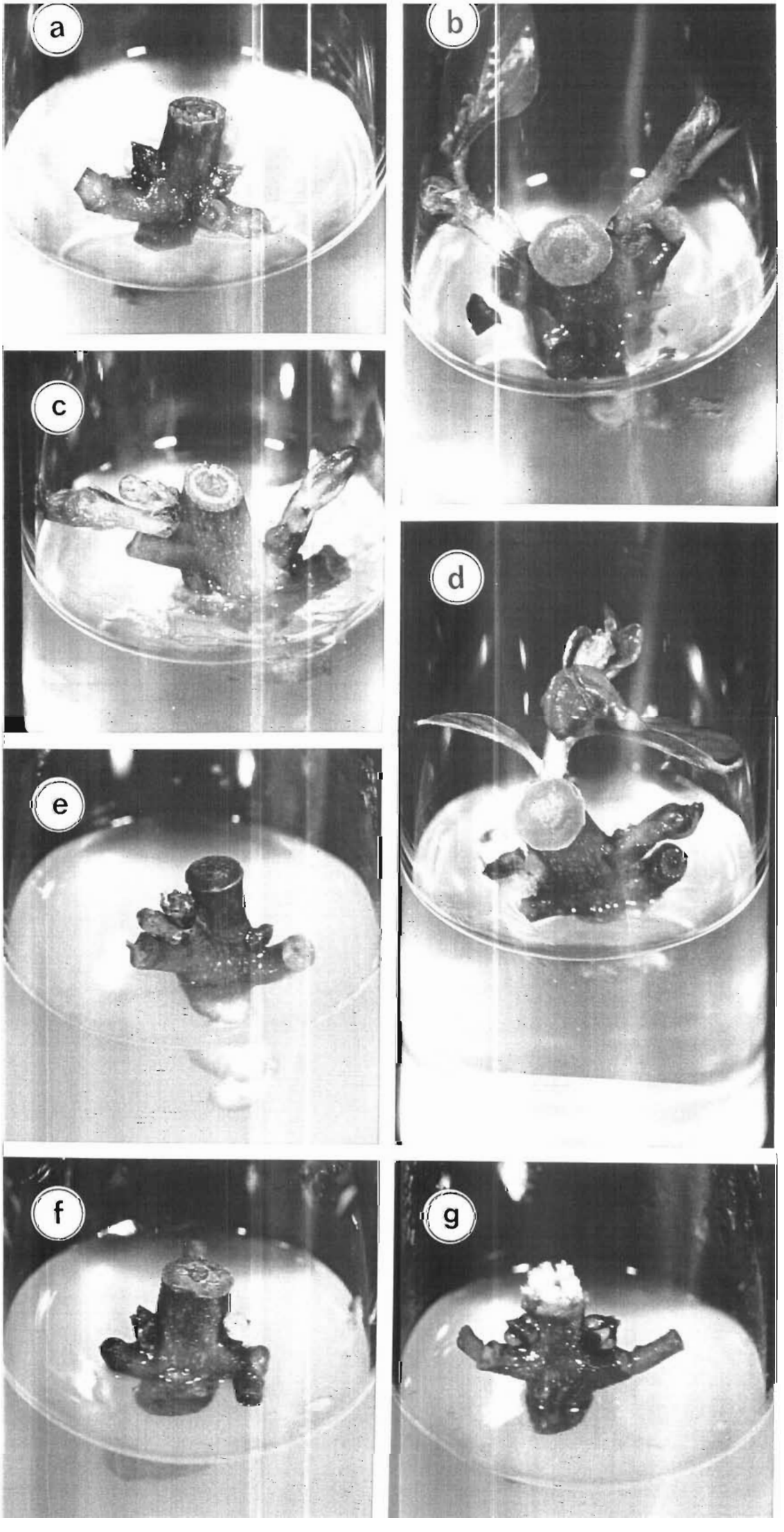
(c) stimulation of two shoots from a single axillary bud (0.5% Kelpak);

(d) elongation of three shoots, one from each axillary bud at that node (0.5% Kelpak);

(e) slightly swollen buds, but unhealthy swelling and callus development at explant base (1.0% Kelpak);

(f) callus development on axillary bud (1.0% Kelpak); and

(g) callus development on top of explant (1.0% Kelpak).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bracteosum shoots however required a high auxin level in order to initiate cell division and organize the root primordia. A two phase procedure (WENT, 1939; THIMANN, 1977; JAMES and THURBON, 1979; SNIR and EREZ, 1980; JAMES and THURBON, 1981) was adopted in rooting the shoots - this was due to the necessity for IBA (1.0 mg.ℓ^{-1}) in initiating *Combretum bracteosum* root formation, with the subsequent root elongation phase being highly sensitive to, and inhibited by, high auxin concentrations (explants sub-cultured onto media containing no hormones, 2 g.ℓ^{-1} activated charcoal to absorb remaining IBA).

The roots developing from the shoot were not substantial, however, it was not considered problematic as this root system was temporary. Plantlets in the *in vitro* environment did not need to use energy developing a large root system, as even with its' single or few short roots it was able to support itself easily and all the nutrients it required were readily available to it. Transplantation of the plantlet into a natural environment led to the generation of a more functional root system, which enabled the absorption of minerals and water from the potting media, as well as providing support to the growing plant.

4.3.3 Meristem Culture

Cultured on suitable medium, meristem-tips may be regenerated into plantlets more quickly than tissues from other sources. Treating the meristem explants with a combination of auxin and cytokinin was the first option considered in attempting to stimulate shoot development from the excised buds. Applying BA in combination with either IAA or NAA induced the meristems to swell and the bud scales surrounding the meristematic zone to open and/or elongate (Plate 12). In some cases, (Plate 12 e) the bud scales opened so wide that they eventually snapped off. Once bud swelling and bud scale elongation had ceased, no further morphological development was observed (Figure 15 and 16). So although the two above-mentioned features were initially considered the first two steps in inducing shoot production, further observations led to the conclusion that this was not the case. All meristem explants placed onto either the MS or WP media containing different concentrations of NAA or IAA and BA were able to stimulate bud swelling and elongation. The morphogenetic activity

stimulated by NAA and IAA were very similar, and it was thought that these auxins played only a small role, if any at all, in stimulating the meristematic activity observed (Figure 15). Bearing in mind that endogenous auxin is probably synthesized by the second pair of youngest leaf primordia (SMITH and MURASHIGE, 1970; RIVERS, 1973; SHABDE and MURASHIGE, 1977) - which remain on the explant, it can be hypothesized that the addition of exogenous auxins may be the limiting factor, in that auxin levels become supra-optimal. This view is however not consistent with results obtained, as the lack of shoot production was also evident in explants which had not been exposed to exogenous auxins. In fact, having undergone bud swelling and scale elongation, the morphogenetic ability of the meristems declined.

Growth and proliferation of shoots in culture is stimulated by cytokinin (MINOCHA, 1987). This is achieved by releasing axillary buds from apical dominance. In combination with auxin, a hormonal balance in favor of cytokinin (1.0 mg.ℓ^{-1} BA) was common to all activity stimulated by exogenous hormone application (Figure 15 and 16). Although in the case of *Combretum bracteosum* meristems the desired response of meristematic activation was not obtained, it was clear that high cytokinin concentrations enhanced the response of the explants to exogenous hormone applications. The presence of cytokinin was therefore considered critical in inducing shoot production. With this high cytokinin level favouring shoot development, caution had to be taken not to jeopardize the health of the meristem explants subjected to BA. It has been suggested that cultures are often overexposed to BA (BIONDI and THORPE, 1982; LEE and WETZSTEIN, 1990), resulting from cytokinin concentrations being too high, due to the use of active cytokinins, and sub-culturing too frequently onto high cytokinin containing media. Stemming from this are physical aberrations (shoot retardation), root inhibition and callus formation during culture *in vitro*, as well as during subsequent culture in the greenhouse or field.

The consistent inability of the cytokinin and auxins alone to encourage shoot development suggested that a key factor had not yet been included in the nutrient media.

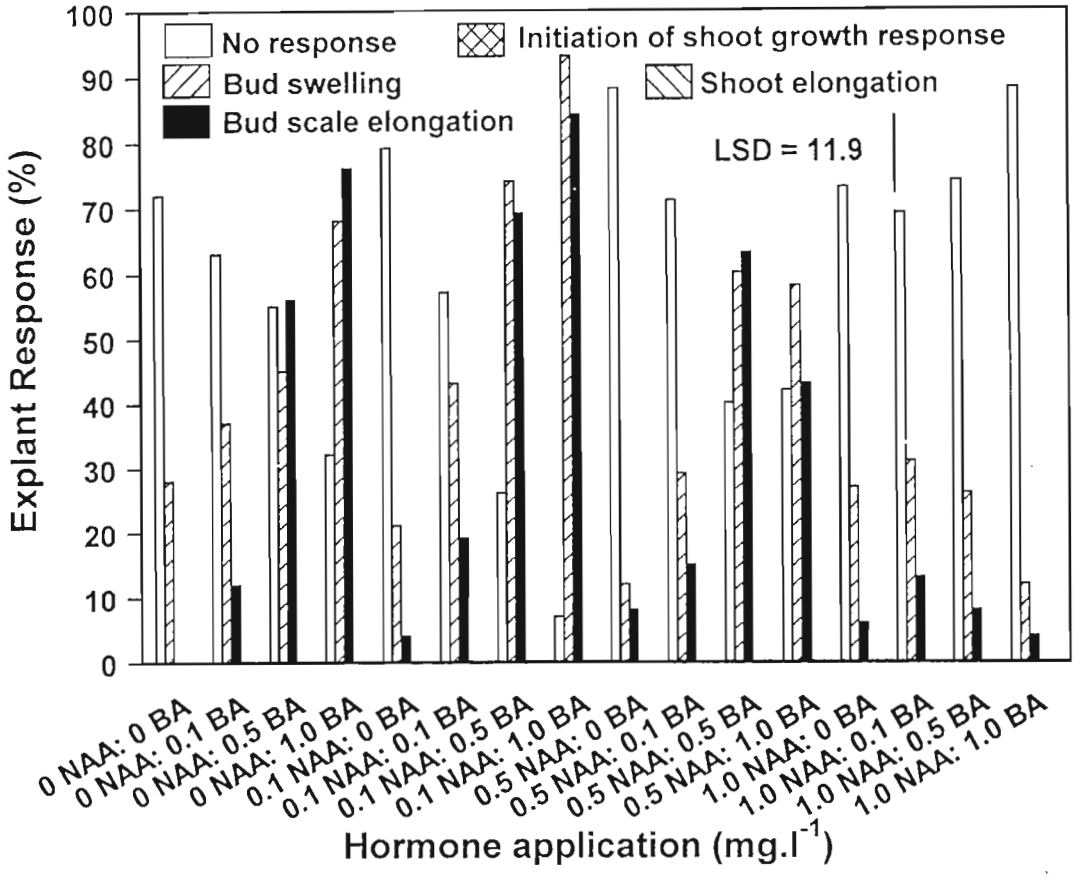


Figure 15: Effect of NAA: BA application on inducing shoot development and elongation in *Combretum bracteosum* meristem explants

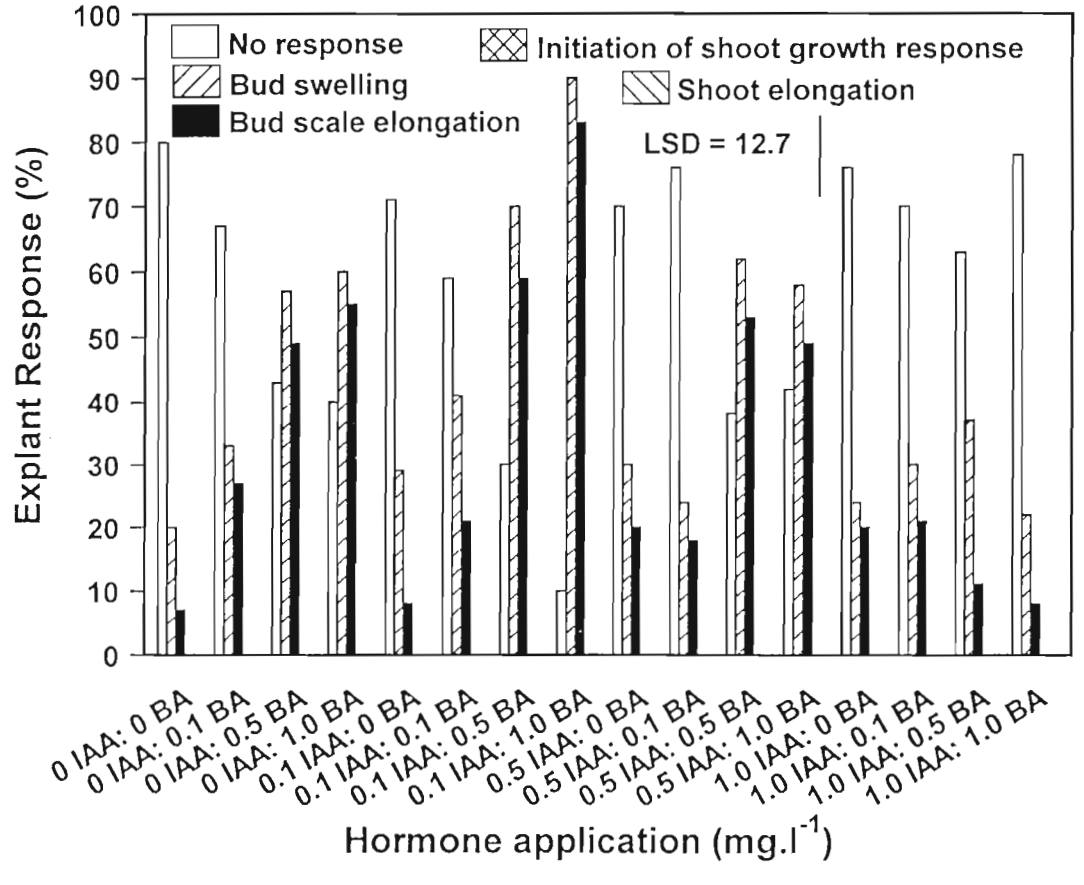


Figure 16: Effect of IAA: BA application on inducing shoot development and elongation in *Combretum bracteosum* meristem explants

Plate 12: Developmental response of *Combretum bracteosum* meristem explants to combined BA: IAA and BA: NAA applications

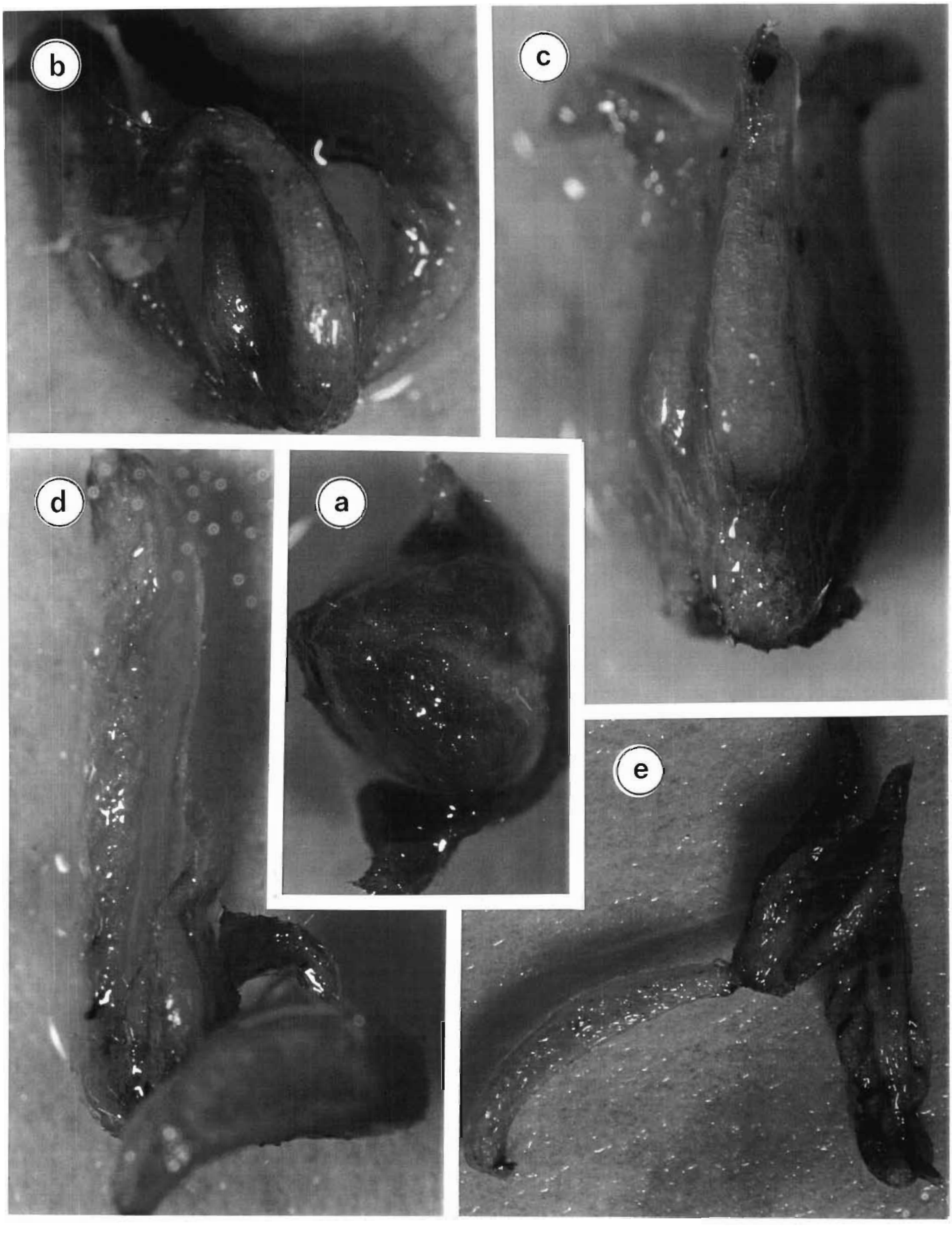
(a) no morphological changes (control, no hormones);

(b) elongation and slight opening of the outer bud scales (0 mg.ℓ^{-1} IAA: 0.5 mg.ℓ^{-1} BA);

(c) elongation, but no opening of all bud scales (0 mg.ℓ^{-1} NAA: 0.5 mg.ℓ^{-1} BA);

(d) elongation of some outer bud scales (0.1 mg.ℓ^{-1} NAA: 0.5 mg.ℓ^{-1} BA); and

(e) elongation of outer buds scales (0.5 mg.ℓ^{-1} NAA: 0.5 mg.ℓ^{-1} BA). Evidence of bud scale opening too far and subsequently snapping off explant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The role of GA_3 in meristem tip culture was first emphasized by MOREL *et al.* (1968). They reported that in *Dahlia,* the presence of 0.1 mg.ℓ^{-1} GA_3 suppressed callusing but favoured better growth and differentiation of the meristem. GA_3 in combination with BA and NAA has also been shown to be essential to raise full plants from excised meristem tips of cassava (*Manihot esculenta*; KARTHA *et al.*, 1974). Other researchers however, have found GA_3 to be without an appreciable effect and at higher concentrations, even inhibitory (SHABDE and MURASHIGE, 1977). Given the fact that a plant's response to exogenously applied gibberellins varies from species to species (WARING and PHILIPS,1981; WELANDER, 1985), its potential morphogenetic influence was not worth ignoring in stimulating shoot development and elongation on *Combretum bracteosum* meristem explants.

GA_3 was however not the answer. The application of 0.1 mg.ℓ^{-1} GA_3 produced results that were not significantly (statistically) different to the control, whereas elongation stimulated by the higher gibberellin levels (0.5 and 1.0 mg.ℓ^{-1} GA_3) was targeting the wrong area. The elongation of the bud scales still substituted for shoot production and subsequent elongation. Exogenous gibberellin application had no effect on shoot initiation or elongation.

Although the use of seaweed products in meristem culture research is limited, the results achieved in other spheres of propagating *Combretum bracteosum* again suggested its possible success. The characteristic swelling of the meristem was suppressed by the Kelpak treatment (0.25% and some 0.5% of commercially available Kelpak solution). The bud scales however opened as before, then subsequently died back, exposing a bright green central portion within the meristem (Plate 13). After four weeks incubation on the 0.25% brown algal extract Kelpak, the central meristematic dome had elongated, thus protruding above the dead bud scales (Plate 13 b, d, e and f). At this stage of morphogenesis, it resembled a developing shoot (Plate 13 b).

At higher Kelpak concentrations (0.5 and 1.0%), energy was not channeled into shoot

initiation and subsequent elongation alone. Elongation of the outer bud scales and swelling of the entire meristem was also characteristic (Figure 17 and Plate 13 c - f). As the bud scales were no longer functional, their elongation was wasteful. Kelpaks' use in *in vitro* *Combretum bracteosum* meristem culture was limited (Figure 17) as none of the explants were able to produce shoots which in turn could be rooted and generated into a marketable plantlet. The target zone of the Kelpak treatments was too broad and the morphological influence it had on the explant was short-lived (shoots only elongated two or three

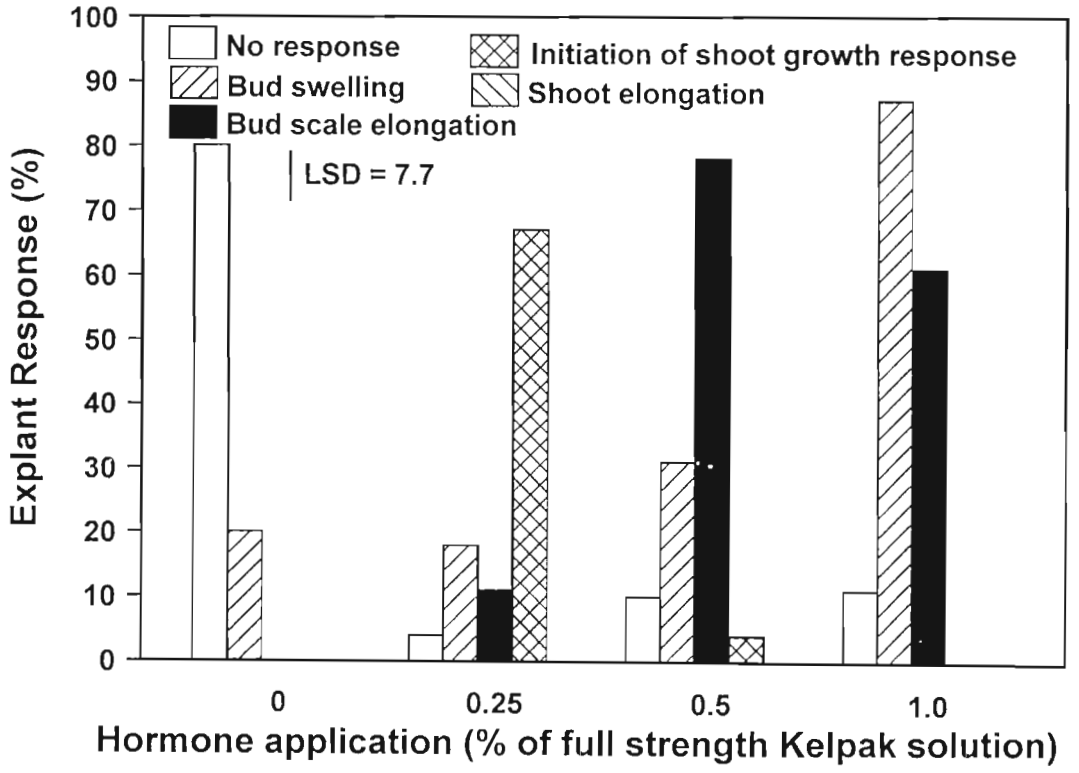

Figure 17: Shoot development from *Combretum bracteosum* meristem explants stimulated by different Kelpak concentrations

millimeters). The subsequent sub-culturing of the meristems onto a hormone-free media (with and without charcoal) as well as onto a media containing GA_3 (0.1 mg.ℓ^{-1}; 0.5 mg.ℓ^{-1} and 1.0 mg.ℓ^{-1}) did not result in any further morphological development.

4.4 Conclusions

Although more time consuming than striking cuttings, promising results have been obtained

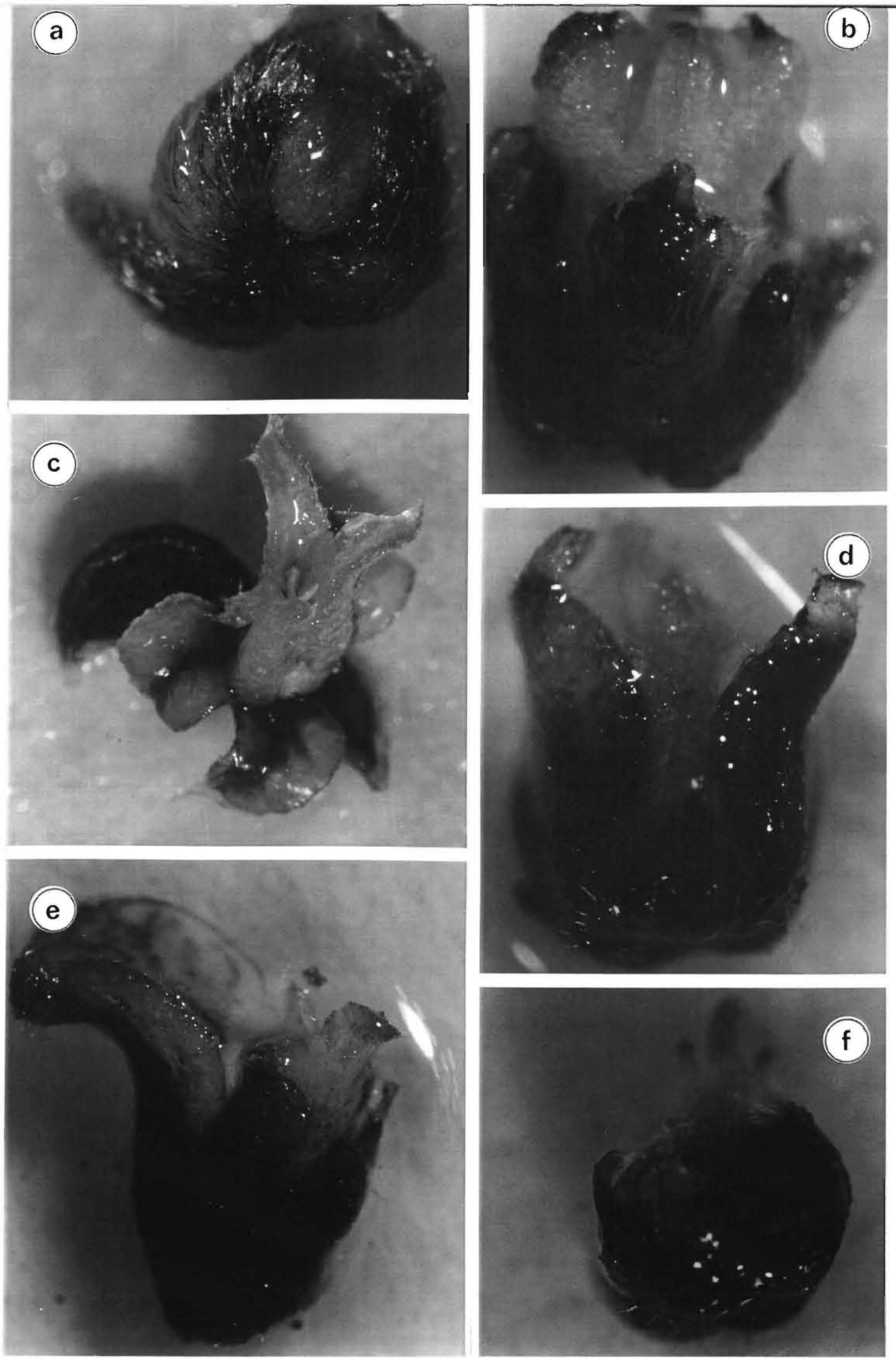

Plate 13: Morphogenetic response of *Combretum bracteosum* meristem explants, stimulated by *in vitro* Kelpak treatments

(a) unresponsive nodal explant placed on media containing no exogenous hormones (control);

(b) shoot initial protruding above outer bud scales (0.25% Kelpak);

(c) slightly elongated central meristematic zone, accompanied by elongating and opening outer bud scales (0.5% Kelpak);

(d) swollen bud and elongating outer bud scales inhibiting central meristematic initials from elongating (0.5% Kelpak);

(e) swollen bud and elongating outer bud scales inhibiting central meristematic initials elongating (1.0% Kelpak); and

(f) swollen bud with no bud scale elongation and the central meristematic dome elongating to protrude above the protective covering structures

from culturing *Combretum bracteosum* nodal explants and germinating the seed *in vitro*. The complex interactions of intrinsic and extrinsic factors in the *in vitro* system, provide endless questions and alternatives which could have been addressed. As producing a healthy plantlet was the goal of this project, only the most important problems encountered during the course of experimentation were considered.

It is only the poor availability of *Combretum bracteosum* seed that may hamper the use of it during *in vitro* germination for commercial propagation purposes. Although germination *in vivo* has proved successful, the establishment of a sterile seedling stock for further multiplication is also desirable. Experimentation has shown that using nodal explants from *in vitro* germinated stock plants, is a rapid and successful method of generating a larger seedling stock. Although more labour intensive, the same is true for the stimulation of axillary shoot elongation where explants had been collected from mature parent plants growing in a natural environment. Here the number of shoots produced per explant was unpredictable. Using a BA: NAA combination, one axillary bud could be stimulated to elongate, whereas the use of Kelpak usually stimulated three or four shoots per node. Once the elongated shoots had been removed from the explant for root initiation, the "parent" explant could be induced to generate another elongating shoot originating from a secondary axillary bud in the same location.

Although not successful in this case, meristem culture shows potential. In being able to determine the "elongation-inducing compound" and subsequently stimulating it to target the central meristematic zone only, the most productive means of propagating *Combretum bracteosum* plants for a commercial market could be elucidated.

Chapter 5
Dwarfing of *Combretum bracteosum*

5.1 Introduction

Pruning techniques such as hand pinching, shaping, and lowering the growth of nursery plants can be replaced for many species by treatment with growth regulators, although their effect depends on the species, the solution, methods of application, environmental conditions, and other factors (GRZESIK, 1989). The tremendous cost associated with manual pruning techniques makes the alternative use of growth regulators very attractive (CREED, 1975). More than two hundred and fifty species and cultivars of plants have been tested with the retardants, and in so doing, the assortment of pot-plants available for commercial retail has been extended (RAUNKOVA, 1989).

The use of chemicals to alter the growth pattern of plants to economic advantage has been a tantalizing objective since the 1930's when the first growth substances, auxins, were discovered. The discovery and development of good, valuable products have been few and mostly economically insignificant (LEVER, 1989) because of the great difficulty of the science involved and the great subtlety of the biological effects sought.

Around 1980 Dutch growers of woody outdoor plants started to sell their products at the flower auction. With a growing interest in visually attractive woody nursery plants produced in containers, emphasis in horticulture and floriculture has been laid on growth inhibitors (JOUSTRA, 1989). The primary objective in this regard, has been to adapt outdoor plants morphologically to suit indoor environments by regulating growth, promoting branching and stimulating flowering. In previous years this goal has been partly achieved by means of 'natural methods' some of which are the use of clay pots, improved lateral branching and flowering. Clay pots have served as growth retardants as there is less evaporation as well as a decrease in the volume of root medium. Improved lateral branching has been achieved

with optimal fertilization, a constant water supply, breeding and protection (GRZESIK, 1989). Infrequent watering also causes stress and inhibition of growth. Long photoperiods stimulate earlier flowering of particular cultivars, while short photoperiods keep some plants shorter and stimulate lateral branching (NITSCH and SOMOGY, 1958 in GRZESIK, 1989).

The use of growth regulators in the cultivation of woody ornamentals is small and development is generally still in the research phase. The few studies done on growth regulator effects on ornamental trees and shrubs (JOUSTRA, 1989) formed the basis of this experimental work, however literature was supplemented with experiments done on fruit tree crops such as peach, apricot, cherry, plum, apple, mango and pear.

Combretum bracteosum grows vigorously, producing large shrubs which are difficult to manage. Neither pruning nor training are fully effective in controlling shrub size and alternative methods of shoot control have been sought. Plant growth retardants offer considerable promise for manipulating the shape, form, and quality of greenhouse ornamentals. Among seventeen well-known plant growth regulators, there are thirteen chemical substances with inhibitory effects (LARSON, 1985). The inhibitory mechanisms of plant growth inhibitors differ from one another, with some being phyto-hormones; abscisic acid and ethylene, whereas others are synthetic chemicals - the growth retardants paclobutrazol (PP333) and AMO-1618.

5.1.1 Chemistry of paclobutrazol (PP333)

The triazoles represent a group of highly active compounds. The growth retarding properties of the triazoles, like many other growth retardants, are largely attributed to interference with gibberellin biosynthesis, hence these compounds are often referred to as "anti-gibberellins".

Paclobutrazol [(2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,3-triaol-1-yl) pentan-3-ol)] (trade names: Bonzi®, Clipper®, Cultar®, Parlay®), is a broad spectrum growth retardant with a range of potential uses (DALZIEL and LAWRENCE, 1984). The precise

features of the molecular structures which confer plant growth regulator activity appear to be related to the stereo-chemical arrangement of the substituents on the carbon chain. Structurally paclobutrazol is a substituted triazol with assymetric carbon atoms (Figure 18) as is produced as a mixture of the 2R, 3R and 2S, 3S enantiomers (SUGAVANAM, 1984; HEDDEN and GRAEBE, 1985). In the cell-free systems the 2S, 3S enantiomer inhibited *ent*-kaurene oxidation more effectively that the 2R, 3R form (HEDDEN and GREABE, 1985).

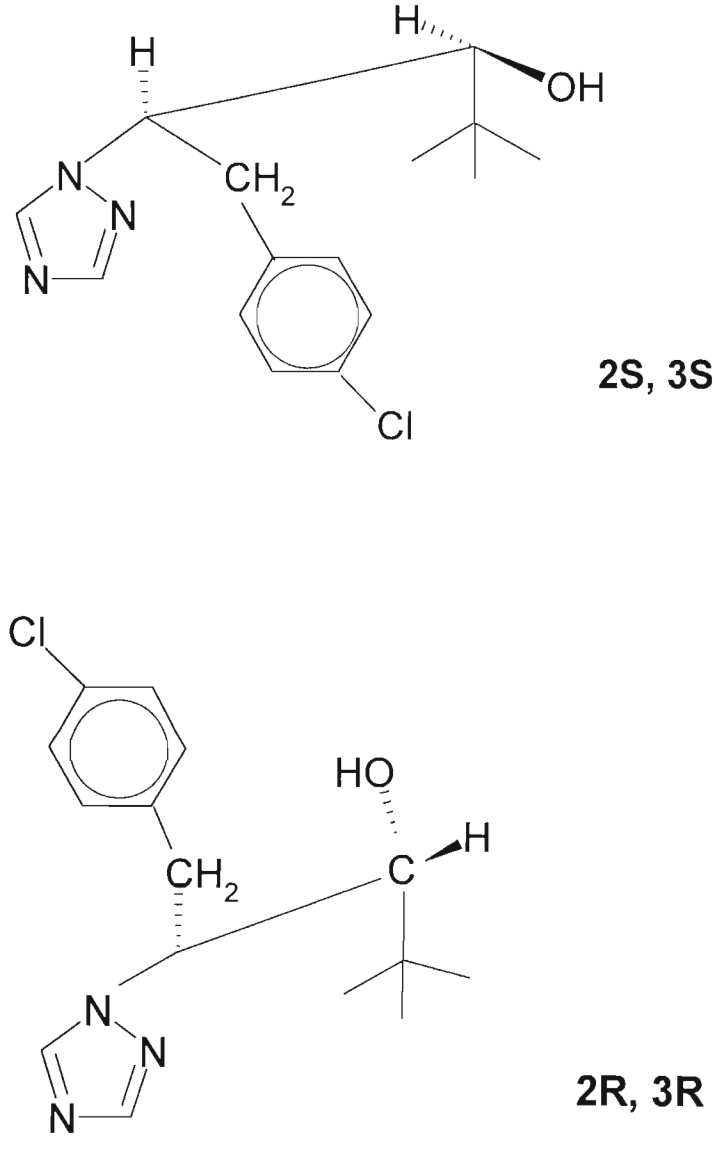

Figure 18: Chemical structure of paclobutrazol, with its two enantiomers

Triazoles inhibit shoot growth in a wide range of species although the dosage required may vary between species or cultivars (DAVIS *et al.*, 1985; GIANFAGNA and WULSTER, 1986 b; HICKMAN, 1986). Paclobutrazol has been effective in controlling growth and promoting compactness of the number of flowering ornamentals (Table 7). 'Cultar®', the growth retardant produced by ICI, seems to be promising for the purpose of dwarfing *Combretum bracteosum*.

Table 7: Ornamental plants dwarfed by paclobutrazol application

Plant name	References
Chrysanthemum morifolium	BARRETT, 1982; BARRETT and BARTUSKA, 1982; McDANIEL, 1983; MENHENNETT, 1984.
Episcia cupreata	STAMPS and HENNY, 1986
Euphorbia pulcherrima	SHANKS, 1980; DAVIS, *et al.*, 1986; McDANIEL, 1986
Freesia hybrida	GIANFAGNA and WULSTER, 1986 a
Hydrangea macrophylla	BAILEY, *et al.*, 1986
Lilium longiflorum	JIAO, *et al.*, 1986
Tulipa	MENHENNETT, and HANKS, 1983

Cultar® known formerly by the code PP333 contains paclobutrazol (TYMOSZUK and MIKA, 1986). As gibberellins play a major role in the control of shoot extension growth it is, as mentioned earlier, generally agreed that paclobutrazol is a particularly effective growth retardant because it is a very potent inhibitor of gibberellin biosynthesis (DALZIEL and LAWRENCE, 1984; HEDDEN and GRAEBE, 1985; QUINLAN and RICHARDSON, 1986).

5.1.2 Proposed mechanism of paclobutrazol action

Gibberellins are synthesized from mevalonic acid via the isoprenoid pathway, and the triazoles specifically inhibit the microsomal oxidation of kaurene, kaurenol, and kaurenal,

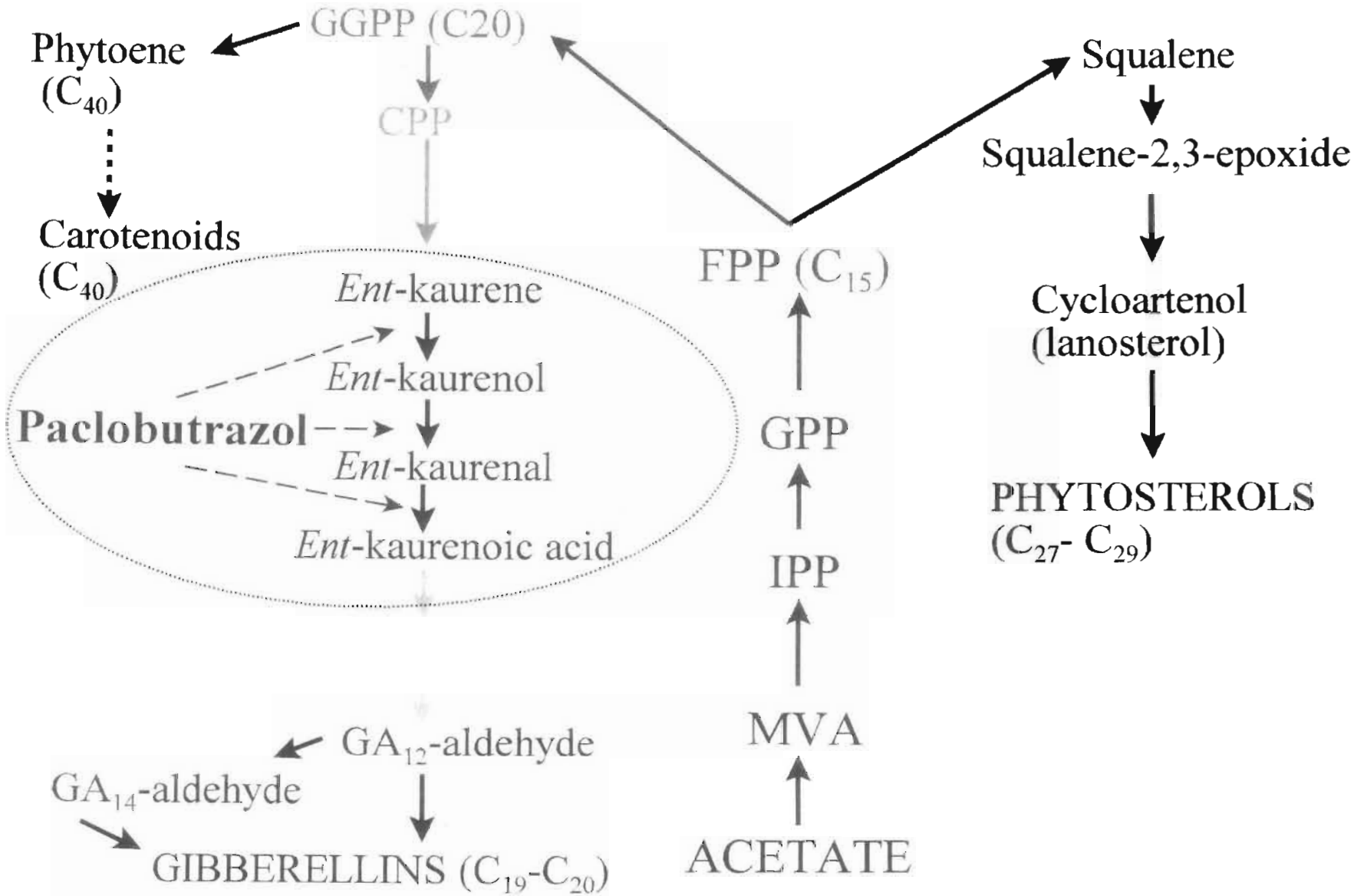

Figure 19: Outline of pathways to gibberellins and phytosterols, showing steps blocked by plant growth retardants Abbreviations: **MVA**, mevalonic acid; **IPP**, isopentenyl pyrophosphate; **GPP**, geranyl pyrophosphate; **FPP**, farnesyl pyrophosphate; **GGPP**, trans-geranylgeraniol pyrophosphate; **CPP**, copyalyl pyrophosphate.

which is catalysed by kaurene oxidase (COOLBAUGH and HAMILTON, 1976; COOLBAUGH *et al.*, 1978; QUINLAN, 1981; HEDDEN and GRAEBE, 1985; STEFFENS and WANG, 1986; DAVIS *et al.*, 1988). These are the same sites of action for a number of other growth retardants, such as ancymidol and tetcyclacis (GRAEBE, 1987). The biosynthetic pathway from mevalonic acid to kaurene and from kaurenoic acid to GA_{12} appears to be unaffected by the triazoles (IZUMI, *et al.*, 1985).

Plants treated with triazoles contain lower quantities of gibberellin-like substances than untreated plants, which is consistent with the proposed primary mode of action (BUCHENUAER and ROHNER, 1981; BUCHENAUER, *et al.*, 1984). Triazole-induced growth inhibition, as well as some of the associated biochemical and physiological changes, can be reversed by the application of GA_3 (DAVIS, 1986), which indicates that the triazoles do not block the activity of either existing endogenous or exogenous GA_3 (CURRY and WILLIAMS, 1983; QUINLAN and RICHARDSON, 1984; STEFFENS *et al.*, 1985 a; WANG *et al.*, 1985; LEVER, 1986; STEFFENS and WANG, 1986). The term "anti-gibberellin" is, therefore, somewhat misleading. These observations however, support the hypothesis that growth inhibition by triazoles is primarily due to reduced gibberellin synthesis.

5.1.3 Transport and metabolism of paclobutrazol within the plant

An important aspect in fully understanding the action of any applied growth regulator, is to obtain information on how the compound is transported and metabolised within the target plant. Such information is useful in devising effective methods of application.

Paclobutrazol is readily taken up and transported throughout plants. QUINLAN and RICHARDSON (1986) applied ^{14}C-paclobutrazol to different parts of apple shoots and found that it was translocated acropetally. They, as well as EARLY and MARTIN (1989), showed that in soil drench applications, ^{14}C activity was distributed predominantly between the roots and leaves and was relatively uniform throughout the remainder of the plant.

P333 applied as a foliar spray, showed that most of the ^{14}C move from the youngest unrolled leaf, whereas no label moves out of the mature leaves. WANG, *et al.* (1986) reported that foliar applied paclobutrazol was not transported to stems or roots. The acropetal movement of PP333 in the stem, was also consistent with the view that paclobutrazol moves in the transpiration stream via the xylem. The presence of small amounts of paclobutrazol in phloem tissue has been ascribed to lateral transport from the xylem (WANG *et al.*, 1986).

LEVER (1986) proposed that a threshold concentration of paclobutrazol needs to be available in the shoot apex to maintain GA biosynthesis suppression. The vascular system behind the growing point may act as a reservoir for the reversibly bound PP333, provided its concentration remains relatively high. Little or no inhibitor reserve was found to be available from foliar or bud treatment, which could indicate why soil applications of triazoles are generally more effective in retarding growth than foliar applications (BARRETT and BARTUSKA, 1982).

5.1.4 Application of paclobutrazol

The effectiveness of a growth retarding chemical is determined, not only by how it is transported from the point of application to the site of action, and the efficiency with which it interferes with the endogenous control system regulating plant growth, but also from the ease with which it is taken up by the plant (KARASZEWSKA *et al.*, 1986; QUINLAN and RICHARDSON, 1986). Several studies have shown that plant growth regulators, when sprayed, drenched or injected can successfully stimulate or retard growth and improve plant-quality under greenhouse as well as field conditions (THOMAS, 1982; WILLIAMS, 1982; MARINI, 1987; WOOD, 1988).

Before using a growth regulator it must be determined carefully which plant-factor should be manipulated, and how the plant-factor should be changed (JOUSTRA, 1989). Uptake and movement studies have shown that in theory root uptake of Cultar® can be an efficient way to maintain a supply of chemical in the growing apex of a young plant. However, in practice factors such as tree size, rooting pattern, water and chemical distribution in the soil, and soil

physical properties may alter the suitability of this method of application. Although much of the chemical from a foliar spray is 'wasted' by leaf interception, shoot uptake sometimes offers a way to get a rapid response to Cultar® (SHEARING and JONES, 1986).

5.2 Materials and Methods

Seeds of *Combretum bracteosum* were germinated under controlled conditions and seedlings were then transplanted into pots containing a potting medium of seedling mix, compost and Umgeni sand, in equal volumetric proportions. The seedlings were watered lightly with tap water daily. A nutrient supplement of Chemicult was applied at three monthly intervals.

Initially, experimentation was carried out in the same greenhouse that was used for the establishment of the seedlings. After twelve months, the plants required more space, so were moved into a shade house. Temperatures in the greenhouse ranged between 18.5°C and 30.8°C; the temperature was not controlled. Daylength (in the greenhouse and shadehouse) and temperature (shadehouse) were those prevailing naturally in Pietermaritzburg (30°E; 30°S) during spring, summer, autumn and winter of 1998-2001.

Eight weeks after germination, the seedlings had reached an adequate height (±10 cm) to commence experimentation. The height of each seedling and its number of fully expanded leaves were recorded.

The test chemical paclobutrazol, [(2RS, 3RS)-1- (4-chlorophenyl)-4,4-dimethyl-2-(1,2,3-triaol-1-yl) pentan-3-ol)] was applied as either a soil drench or a foliar spray. When the plant growth retardant solution with fungitoxic sterol-inhibitory properties was applied as a soil drench, it was important to ensure that the potting medium was totally saturated, yet with minimal losses due to drainage. The application of forty millilitres PP333 was sufficient in the first twelve months; subsequent treatments were increased to one hundred millilitres per plant as the plants had been repotted into bigger containers.

With the foliar spray it was important to ensure that the active ingredients did not contaminate the potting mix, as the subsequent uptake by the roots would distort results. The soil was therefore covered with aluminium foil. The paclobutrazol solution was sprayed completely and uniformly onto the individual plants. The spray was fine and the volume applied considered sufficient when the spray volume exceeded the plant retention volume ie. sprayed to drip point (± 50 ml).

The commercially available Cultar® solution was diluted until the active ingredient (paclobutrazol) it contained, measured 50, 100 and 250 mg.ℓ^{-1}. These three concentrations were used in soil drench experiments. The foliar spray experiment made use of two of those

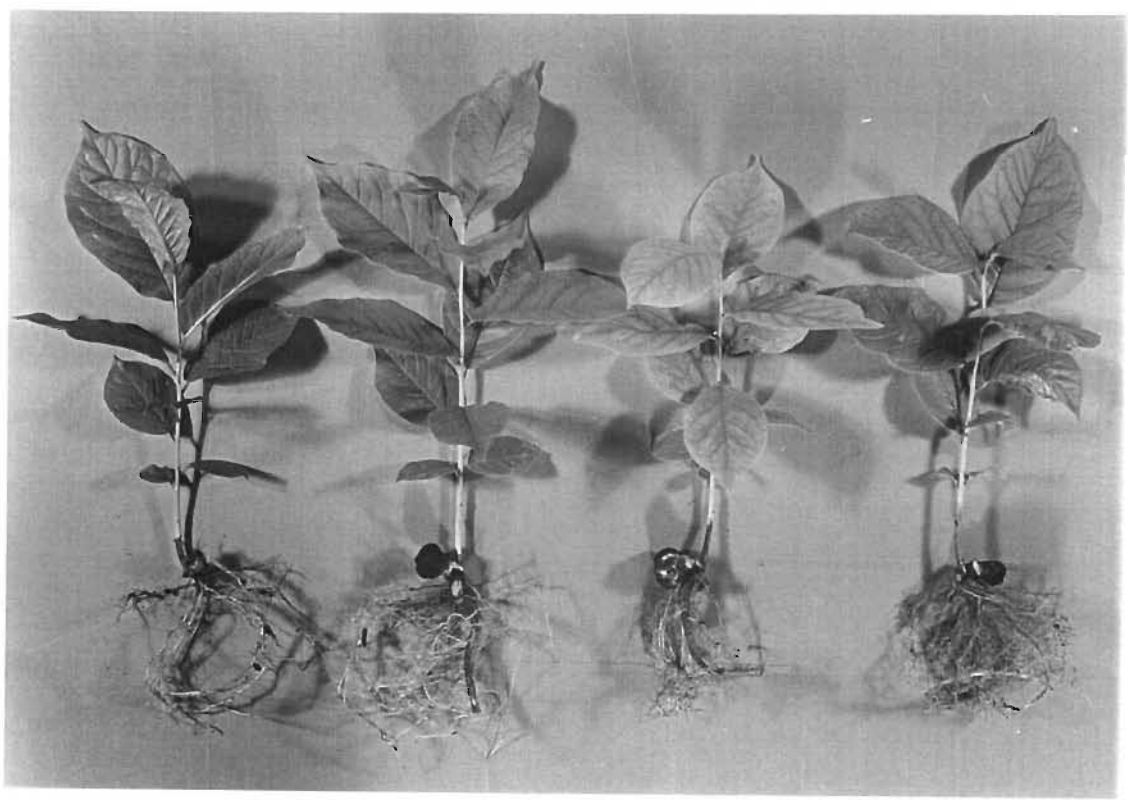

Plate 14: Appearance of eight-week-old *Combretum bracteosum* seedlings, prior to treatment with paclobutrazol

concentrations (containing a surfactant, Tween 80), namely 50 and 100 mg.ℓ^{-1}. The Cultar® emulsion concentrate was diluted with tap water. The control consisted of tap water (and Tween 80 in the foliar spray treatments) only. These applications were repeated five times,

at three day intervals. The same paclobutrazol treatments were applied annually.

Six months after the first paclobutrazol treatment, the seedlings that had been treated with a 250 mg.ℓ^{-1} PP333 soil drench were foliar sprayed with a once off treatment of 100 mg.ℓ^{-1} paclobutrazol. This was in an attempt to increase the leaf size, as while the seedlings were dwarfed sufficiently, the leaves were uncharacteristically small (Plate 16 h).

Six months after the initial paclobutrazol application, the following growth parameters were recorded: plant height; fresh and dry mass of leaves, stems and roots; leaf area; leaf number and leaf chlorophyll content. As this is a destructive analysis, only a representative number of plants (two) of each treatment, was harvested. Subsequent analysis was recorded every six months. The experimental design was a randomised complete block with twenty replicates per treatment. Statistical analysis (ANNOVA, general analysis of variance) was done on all data obtained in order to determine whether relationships existed between treatment types over time, as well as to ascertain whether there was any interaction between the treatment type and time. Standard error and L.S.D. were also calculated.

5.2.1 Plant tissue analysis

a) Plant height

Shoot length was determined by measuring from the root collar to the highest point the shoot reached unsupported.

b) Fresh mass.

Tissue was massed immediately after harvesting, allowing for minimal moisture loss.

c) Leaf area.

Measurement of leaf area was conducted by removing the leaves from the plants and passing them through a Li-Cor 3100 leaf area meter. Readings accurate to two decimal points of a square centimetre were obtained for each plant. Averages were calculated to represent each treatment.

d) Dry mass.

Tissue was dried in an oven (60°C) until it reached a constant mass. Approximately 5 days.

e) Chlorophyll determination.

Chlorophyll was extracted from leaf segments according to the method of INSKEEP and BLOOM (1985). Leaf tissue was obtained from the terminal leaves of each plant being harvested. Each sample was weighed accurately to obtain 0.1 gram fresh weight. These leaf samples were homogenised using a mortar and pestle and extracted overnight in 10 ml N,N-dimethylformamide. The resultant homogenate was vacuum filtered through Whatman No. 1 filter paper. N,N-dimethylformamide (DMF) is a very convenient solvent for chlorophyll extraction since it is effective on intact plant parts and the chlorophyll remains stable.

Using a ten millimetre cuvette, absorbance was measured using a Beckman DU-64 spectrophotometer at 664.5 nm and 647 nm (maximum for chlorophyll a and b respectively). Absolute chlorophyll concentrations (chlorophyll a and b), expressed in milligrams per litre, were calculated according to INSKEEP and BLOOM (1985).

f) Histological studies.

Histological studies were performed on sectioned stems, roots and leaves, at harvesting. This was to see the effects of the growth regulator at a cellular level. The young untreated plants, harvested in 1998 were small enough to be fixed in Epon resin. Resin embedding was achieved by initially fixing the tissue in glutaraldehyde buffered with sodium cacodylate, followed by three washes in buffer and post fixed with osmium tetroxide. The tissue was then dehydrated in a graded alcohol series and impregnated with Epon resin as outlined in Table 8.

After trimming, ultra-thin sections of the specimen were cut by a Reichert Jung Ultracut E microtome using glass knives made on a LKB knife maker. These sections were mounted onto glass slides and stained with Ladds multiple stain. The

sections were given a final rinse in double distilled water and dried. A drop of 100% Epon resin covered each specimen and sealed the coverslip to the glass slide. The *Combretum bracteosum* root, stem and leaf sections were then examined under an Olympus BH-2 light microscope.

The rapid growth of the *Combretum bracteosum* plants soon made resin embedding unsuitable as the resin was no longer able to penetrate the thick stem and roots. After only twelve months since their first PP333 treatment, the simpler technique of wax embedding proved a more suitable option. At each collection date, root, stem and leaf material was fixed, dehydrated and infiltrated as described in Table 9. This technique involved fixing the specimens in a FAA (formalin: acetic acid: alcohol) solution for one week (to ensure total impregnation of FAA into the thickened stem and root sections) and 24 hours for the thinner leaf samples. Transferral of the fixed specimens into a graded tertiary-butanol series (water: ethanol: tertiary butanol) ensured complete dehydration and aided subsequent wax infiltration (Table 9). Once the material was completely impregnated with wax, each specimen was embedded in pure wax and refrigerated for approximately two hours. The wax blocks in which the specimens were embedded were then trimmed, so that the correct thickness and orientation could be obtained.

Once the sections (±10μm thick) had been cut with a Leitz Wetzlar microtome, they were mounted onto glass slides coated with Haupt's adhesive. A drop of 3% FAA was placed onto each slide. Ribbons of sections were floated onto the FAA, after which they were stretched and dried at 40°C. In order to identify the different plant tissues correctly, the sections were stained with Safranin and Fast Green (Table 11). Prior to staining, the sections had to be rinsed in xylene twice (for three minutes each) in order to remove the wax. The staining procedure is outlined in Table 10. The specimens were viewed under a light microscope.

Table 9: Preparation routine for wax embedding

Procedure	Time
Fixation	
Fix in FAA (Formalin: acetic acid: alcohol)	
FAA 96% ethanol Acetic acid 37% formaldehyde Distilled water	Minimum 24 hours
Dehydration	
Dehydration in graded tertiary-butanol series (water: ethanol: tertiary butanol)	
45: 45: 10	1 hour (room temperature ±20°C)
30: 50: 20	12 hours (±20°C)
15: 50: 35	1 hour (±20°C)
15: 40: 55	1 hour (±20°C)
0: 25: 75	1 hour (±20°C)
0: 0: 100	2 hours (40°C)
0: 0: 100	18 hours (40°C)
Infiltration	
Infiltrate the dehydrated tissue with wax	
Tertiary butanol: Liquid paraffin (50: 50)	24 hours (40°C)
Liquid paraffin	12 hours (40°C)
Liquid paraffin	12 hours (40°C)
Liquid paraffin and a few wax pellets	12 hours (40°C)
Liquid paraffin and wax pellets	24 hours (60°C)
Pure molten wax	48 hours (60°C)

Table 10: Safranin and Fast Green Staining procedure

Procedure	Time
Xylene: alcohol (50: 50)	1 minute
95% alcohol	30 seconds
70% alcohol	30 seconds
Safranin stain	20 seconds
95% alcohol	30 seconds
Absolute alcohol	1 minute
Absolute alcohol	1 minute
50% alcohol	1 minute
50% xylene	1 minute
Fast Green	Few seconds
50% xylene: 50% alcohol	30 seconds
100% xylene	1 minute

Table 11: Stained plant tissues are highlighted by a specific colour for easier identification

Tissue type	Colour
Cellulose	Blue-green
Cytoplasm	Blue-green
Lignin	Red
Suberin	Red/orange
DNA	Red/purple
Tannins	Red/brown

5.3 Results and Discussion

Growth retardants are highly specific. There is no obvious correlation between taxonomic classification and plant response to a particular compound. Even different cultivars of the same species vary greatly in responsiveness to the applied plant growth regulator. Paclobutrazol (PP333), has retardant activity on a broad range of species. Its potential for controlling excessive vegetative growth has been demonstrated on a number of fruit crops: apples (WILLIAMS, 1982), peaches (MARINI, 1987) and pecans (WOOD, 1988). PP333 controls vigorous vegetative growth, leading to benefits in reduced pruning. It also results in a thicker leaf together with an intensified green colour, increased flower bud induction, yield and fruit quality improvements (EDGERTON, 1986; OGATA *et al.*, 1986; SHEARING and JONES, 1986; GRZESIK, 1989). Due to triazoles being highly active in retarding growth, excessive PP333 application had to be avoided in order to prevent delayed flowering and/or excessive dwarfing (Plate 16 h).

The physiological age of plants and the timing of applications are important factors for many species, with the exception of evergreens. Other general rules which are especially important to many ornamental shrubs and trees are treatment technique and climatic conditions. The time of application also depends on frequency of application, the concentration and the duration of the presence of exogenous growth regulators in the plant (GRZESIK, 1989). The wide variance in the climatic conditions prevailing in different geographic locations could cause a variation in results, even though the solutions and the method of application may be the same. Geographical location therefore has an important effect and has to be considered closely when using growth regulators. Differences in natural light, temperature and other environmental conditions encountered at the experimental site may result in the recommended protocol being manipulated to best suit the circumstances in question. As an example, in the United States higher concentrations of PP333 are recommended than in Europe (GRZESIK, 1989).

5.3.1 Effects of paclobutrazol on *Combretum bracteosum* plants

Results obtained after a mere six months highlight the fact that paclobutrazol has considerable potential for modifying plant architecture (Plates 15 and 16). The rapid effects of this PGR and the fact that it is known to exhibit dwarfing effects for more than a three year period (especially with the soil application (ELFING and PROCTOR, 1986)), make it highly desirable to the horticulturalist.

Cultar® was effective in suppressing *Combretum bracteosum* shoot growth, although in the first two years (1998/1999) there was usually a preferential inhibition of lateral shoot formation over terminal shoot formation (Plates 15 a - f, and 16 a - h). This undesired phenomenon was however overcome during the third year (soil drench) or fourth year (foliar spray) of treatment, where lateral shoots developed and a more shapely, bushy dwarfed plant resulted (Plate 16 k - l and Plate 15 j - l respectively). This change in growth pattern is reported to be favourable for flower induction (BUBÁN, 1986).

As expected, the different techniques of Cultar® application provided different results with regard to the degree of dwarfing (Figure 20 a and b). In order to fully understand these effects, it is important to consider the methods of paclobutrazol application and uptake in more detail.

A frequent limitation to the general use of plant growth regulators, is the high degree of inconsistency in performance. The basis for the inconsistent response is not clear. However, when looking at PP333 foliar spray application alone, irregularities start at the complex stage of application, where numerous inter-dependant components indirectly determine the success of this technique. For this reason it has been the focus of extensive research (BUKOVAC *et al.*, 1986). Uptake from foliar spray is from a very small portion of the tree canopy - the young green shoots, with little uptake from the woody stems. The small size of the target, coupled with very limited translocation of paclobutrazol from leaves, highlights the need for good spray coverage of the shoots in the tree canopy (QUINLAN and

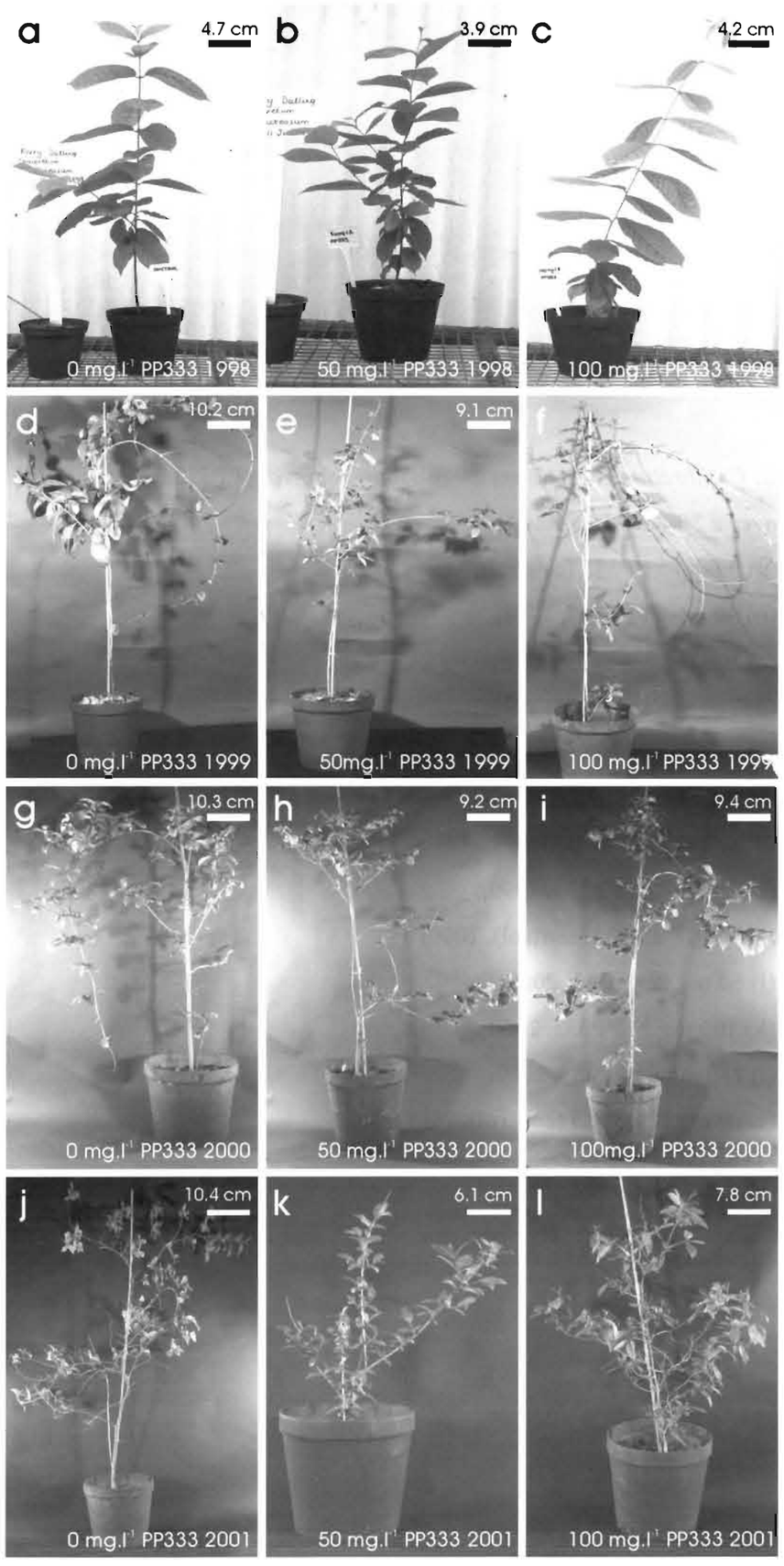

Plate 15: ***Combretum bracteosum*** **morphology following PP333 foliar spray treatments over a 36-month period**

(a) 0 mg.ℓ^{-1} PP333 (control), 1998;

(b) 50 mg.ℓ^{-1} PP333, 1998;

(c) 100 mg.ℓ^{-1} PP333, 1998;

(d) 0 mg.ℓ^{-1} PP333 (control) 1999;

(e) 50 mg.ℓ^{-1} PP333, 1999;

(f) 100 mg.ℓ^{-1} PP333, 1999;

(g) 0 mg.ℓ^{-1} PP333 (control), 2000;

(h) 50 mg.ℓ^{-1} PP333, 2000;

(i) 100 mg.ℓ^{-1} PP333, 2000;

(j) 0 mg.ℓ^{-1} PP333 (control), 2001;

(k) 50 mg.ℓ^{-1} PP333, 2001; and

(l) 100 mg.ℓ^{-1} PP333, 2001

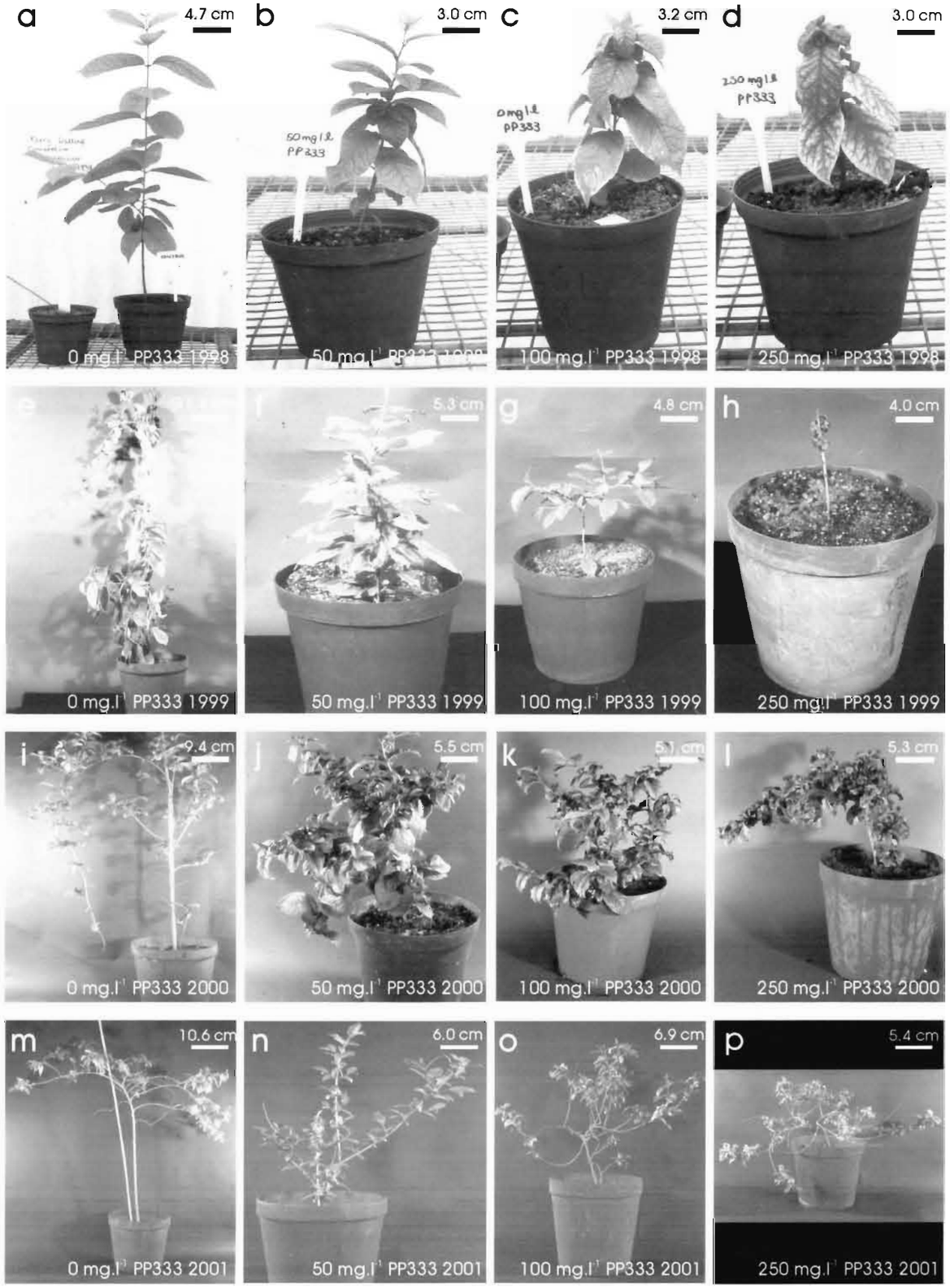

Plate 16: ***Combretum bracteosum*** **morphology following PP333 soil drench treatments over a 36-month period**

(a) 0 mg.ℓ^{-1} PP333 (control), 1998;

(b) 50 mg.ℓ^{-1} PP333, 1998;

(c) 100 mg.ℓ^{-1}PP333, 1998;

(d) 250 mg.ℓ^{-1} PP333, 1998;

(e) 0 mg.ℓ^{-1} PP333 (control), 1999;

(f) 50 mg.ℓ^{-1} PP333, 1999;

(g) 100 mg.ℓ^{-1} PP333, 1999;

(h) 250 mg.ℓ^{-1} PP333, 1999;

(i) 0 mg.ℓ^{-1}PP333 (control), 2000;

(j) 50 mg.ℓ^{-1} PP333, 2000;

(k) 100 mg.ℓ^{-1} PP333, 2000;

(l) 250 mg.ℓ^{-1} PP333, 2000;

(m) 0 mg.ℓ^{-1} PP333 (control), 2001;

(n) 50 mg.ℓ^{-1} PP333, 2001;

(o) 100 mg.ℓ^{-1} PP333, 2001; and

(p) 250 mg.ℓ^{-1} PP333, 2001

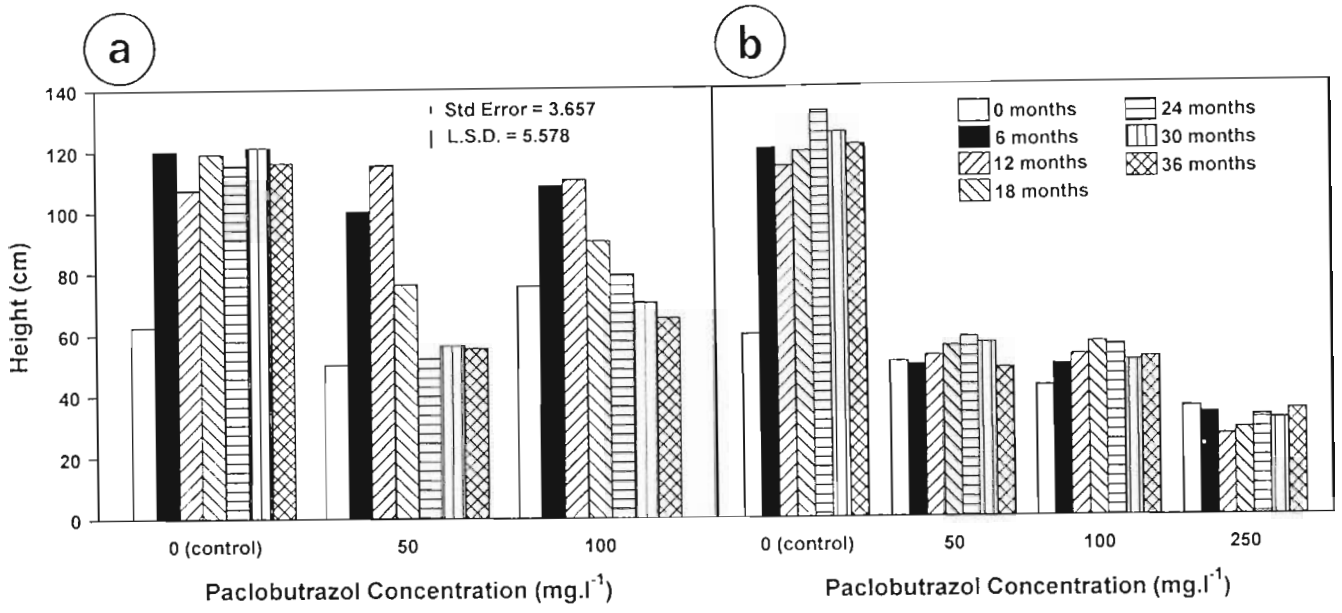

Figure 20: Effects of PP333 on the height of *Combretum bracteosum* plants over a 36-month period (a) foliar spray; (b) soil drench

RICHARDSON, 1986). Foliar sprays were therefore most likely to be effective when the chemical supply to the new green shoots was renewed frequently, with sequential applications having generally been found to be more effective than a single high dose. This is primarily due to the relatively rapid depletion of the chemical in the shoot apex, as the shoot develops (SHEARING and JONES, 1986).

In an attempt to minimize inconsistencies in foliar spray paclobutrazol application, the importance of the spray delivery system in the performance of growth regulators must be emphasized. Some of the more significant factors that should be considered in this regard are:

- the active ingredient
- formulation of the active ingredient
- spray solution characteristics
- droplet formation
- spray pattern characteristics
- transport of droplets to the target

- target definition and characteristics
- environmental parameters during spray application and drying
- spray droplet: leaf surface interaction
- spray deposit transformation
- penetration of the active ingredient; and
- translocation of the active ingredient to the reaction site (BUKOVAC *et al.*, 1986).

The retention of a droplet on a plant surface is the first of numerous complex interactions leading to deposit formation. Physio-chemical characteristics of the active ingredient and formulation, leaf surface morphology and chemistry, the pH of the treatment solution, the environmental (microclimate) conditions during droplet drying, the absorption period, and the properties of the wetting agent all play an important role in regulating the absorption rates of the applied plant growth regulator (BUKOVAC *et al.*, 1986; BEN-TAL and WODNER, 1993).

Factors which improve the uptake of paclobutrazol by the shoot were expected to improve the effectiveness of spray treatments. As the addition of surfactants to paclobutrazol sprays applied to orchard trees had been shown to greatly enhance the effects of the growth regulator in suppressing shoot growth, presumably by solubilizing the paclobutrazol and extending the uptake period (JONES *et al.*, 1989), they were incorporated into foliar spray solutions applied to *Combretum bracteosum* plants. The efficiency of spray application and adoption of a sequential spray regime incorporating an appropriate surfactant system were, therefore, factors which influenced the growth-controlling effect of paclobutrazol sprays greatly.

The interaction of spray droplets with the plant surface plays a critical role in the spraying process (BUKOVAC *et al.*, 1986). The morphology and chemistry of plant surfaces play a critical role in wettability and hence droplet retention. Surface features like venation and

surface contours are associated with spray retention and may provide channels for spreading of liquids on plant surfaces (BUKOVAC *et al.*, 1986). The spray retained per unit area of surface establishes the dose of plant growth regulator available for penetration. This interaction takes on greater significance for low-volume spraying where the liquid is atomized and deposited as a large number of small discrete droplets on the plant surface. Whether a droplet is retained or reflected (bounces off) and the extent of droplet spreading, will be a function of this interaction (BRUNSKILL, 1956; SPILLMAN, 1984).

Performance of growth regulator sprays may be affected dramatically by the concentration and spray volume in which they are delivered. Non-uniform deposition of the spray over the tree is commonplace. Even though growth substances are translocated within plants, for most, the response appears to be localized to an area near the treatment site, suggesting that uniform coverage is important (BUKOVAC *et al.*, 1986). What complicated matters further was that the degree and uniformity of coverage needed for optimum growth regulator performance has not been established.

Paclobutrazol can also be absorbed by the root when it is applied directly to the soil by a soil drench in the tree canopy area (ELFING and PROCTOR, 1986). TUKEY (1986) stated that soil drenches had a stronger effect than a foliar wood spray in reducing shoot growth, with the response from root uptake being most pronounced in the year after treatment. However, determining the optimal rate for soil application of PP333 was difficult, as levels change for different plant species, different ages and soil types (OGATA *et al.*, 1986). LEVER (1986) indicated that the solubility of paclobutrazol in water is low (30 ppm) and its half life in soil varies considerably, but usually is between 3 and 12 months. Movement in soil is also low and is dependent upon soil water movement and the absorption coefficient of the particular soil type. Laboratory studies showed paclobutrazol movement in the soil also to be related to organic matter content, clay content, and cation exchange capacity. Due to paclobutrazol being relatively immobile in soil, uptake via roots will be dependent upon relative proximity of the chemical to the roots.

Liquid application around the tree trunk and the downward movement of paclobutrazol is facilitated because the soil in the immediate area of the crown is often more loose and friable than the soil surface further out from the trunk - as well as there frequently being a slight fissure between the tree trunk at the soil line. These conditions, which tend to promote downward penetration or movement of the applied liquid, brings the paclobutrazol into direct contact with the fibrous root zone (TAYLOR, 1980). Transpiration by leaves is required to ensure the movement of the chemical upwards through the xylem to the meristematic regions (QUINLAN and RICHARDSON, 1986). As paclobutrazol is dominantly xylem mobile, it may mot move to the deeper parts of the root system, leaving rooting depth unaffected. With relatively high levels of the growth regulator coming in direct contact with this zone, absorption is facilitated. As PP333 is also readily tied up by organic matter in the soil, its movement from the soil surface to the sub-surface areas where the majority of the root absorbing surface is located, is limited, in turn effecting optimal dwarfing results (EDGERTON, 1986).

After growth retardant application, there is a characteristic delay period prior to the plant exhibiting growth retardation (DAVIS, 1988). The extent of the delay depends on the method of application (eg. foliar spray, soil drench), transpiration rate and degree of vascular binding, amount of growth retardant reaching the leaves versus the growing points, the level of endogenous gibberellins at the time of treatment, and the time of treatment (season of the year as well as developmental stage of the plant). BLAKE and QUINLAN (1993) also showed that varying the rate of sap flow through the shoot segment also influenced the level of retention, with more paclobutrazol being retained by shoot tissues at lower flow rates.

Dwarfing effects on *Combretum bracteosum* plants were noticeable within four weeks of the initial soil drench treatment, with a reduction in plant height being noted. The desired and continued dwarfing effect induced by foliar sprayed PP333 however, only became apparent after eighteen months, with the suppression of vigorous lateral branch growth. Within two

months of the first foliar spray treatment, visual indications were that the foliar spray technique had been as effective in dwarfing *Combretum bracteosum* plants as the soil drench treatment. This growth restriction was however short-lived as on harvesting plants at six and twelve months their height, was much greater than that of the soil drench-treated plants (Figure 20).

The main effect of the foliar spray treatment within the first eighteen months was to reduce the scandent habit of the treated plants. Plate 15 (d, g and j) shows the untidy, undesirable appearance of the untreated (control) plants. Concentrations of 50 mg.ℓ^{-1} in 1999 (Plate 15 e) displayed a lack of twining and clinging shoots. Higher levels of paclobutrazol (100 mg.ℓ^{-1}) did not follow suit, with its' habit looking very much like that of the control (Plate 15 f and d respectively). Initially, a correlation between internode length and plant height was noted in foliar sprayed plants. Up until eighteen months after initial paclobutrazol treatment, internode length and plant height increase were both unaffected by foliar spray treatments., with measurements of up to fifteen centimetres being recorded for the former. Control plant internode lengths measured approximately 7 cm less than the treated plants. The height of the treated plants followed the same trend, where after treatment their recorded height was greater than the untreated control plants. At eighteen months, and most harvesting intervals subsequent to that, a reduction in both internode length and plant height[2] for treated plants was noted, highlighting the proposed interaction between paclobutrazol and GA_3 synthesis (the latter is directly related to internode elongation). This was definitely an effect of the dwarfing agent as the control plants continued to retain a constant average plant height of about 117 cm and internode length of 8 - 10 cm.

Two years after initial treatment (24 months), paclobutrazol applied to the leaves resulted in reduced plant height which was accompanied by an improvement in appearance. This

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[2]Reduced or decreased plant height or internode length referred to in the text is not literal, but in this case defined as a reduced growth rate. The height of the plants therefore does not decrease, but the amount of growth recorded over a specific time period is inhibited.

was brought about by the increase in lateral branch number, with the number of leaves on each branch increasing too. This made the plant habit appear less tatty and bare than before. Although the height of the 50 mg.ℓ^{-1} PP333-treated plant remained the smaller of the two foliar spray treatments, its' height remained constant over the last eighteen months of the experimental period. The growth rate of 100 mg.ℓ^{-1} PP333-treated plants on the contrary continued at an increasingly slower, rate for the duration of the experiment (Figure 20 a).

The height of soil drenched plants was affected immediately after treatment irrespective of paclobutrazol concentration. The height of the affected plants remained the same over the entire experimental period, highlighting the fact that the active ingredient was able to maintain an uninterrupted dominance over other growth promoting factors. Plant habit was severely modified by a paclobutrazol soil drench, with a more compact, bushy shrub resulting (Plate 16). Variation in dwarfing effects brought about by different PP333 concentrations were both visually and statistically significant for the 250 mg.ℓ^{-1} application only. These high levels of paclobutrazol were detrimental to the plant in the early stages of experimentation, with no lateral branch formation, very little leaf development and internodes measuring only a few millimetres in length (Plate 16 h). Over time (and with the substitution of a single soil drench application for foliar spray for the 250 mg.ℓ^{-1} PP333 soil drench plants in 1999 only - in order to encourage a leaf area increase), lateral branches developed and internode length increased, enabling plants to take on a more compact and bushy appearance. There was no significant difference between the 50 mg.ℓ^{-1} and the 100 mg.ℓ^{-1} soil drenched plant height. Differences between the control and treated plants were also statistically significant (Figure 20 b).

PGR's generally reduced the total cross-section area and modified tissue development in the stem. Paclobutrazol application (especially soil drench treatments) appeared to reduce the number and diameter of xylem vessels with increasing concentration (Plate 17 and 18). As the active dwarfing ingredient would have been transported to the aerial plant parts via the xylem in soil drench-treated plants, morphological changes as a result of PP333 binding

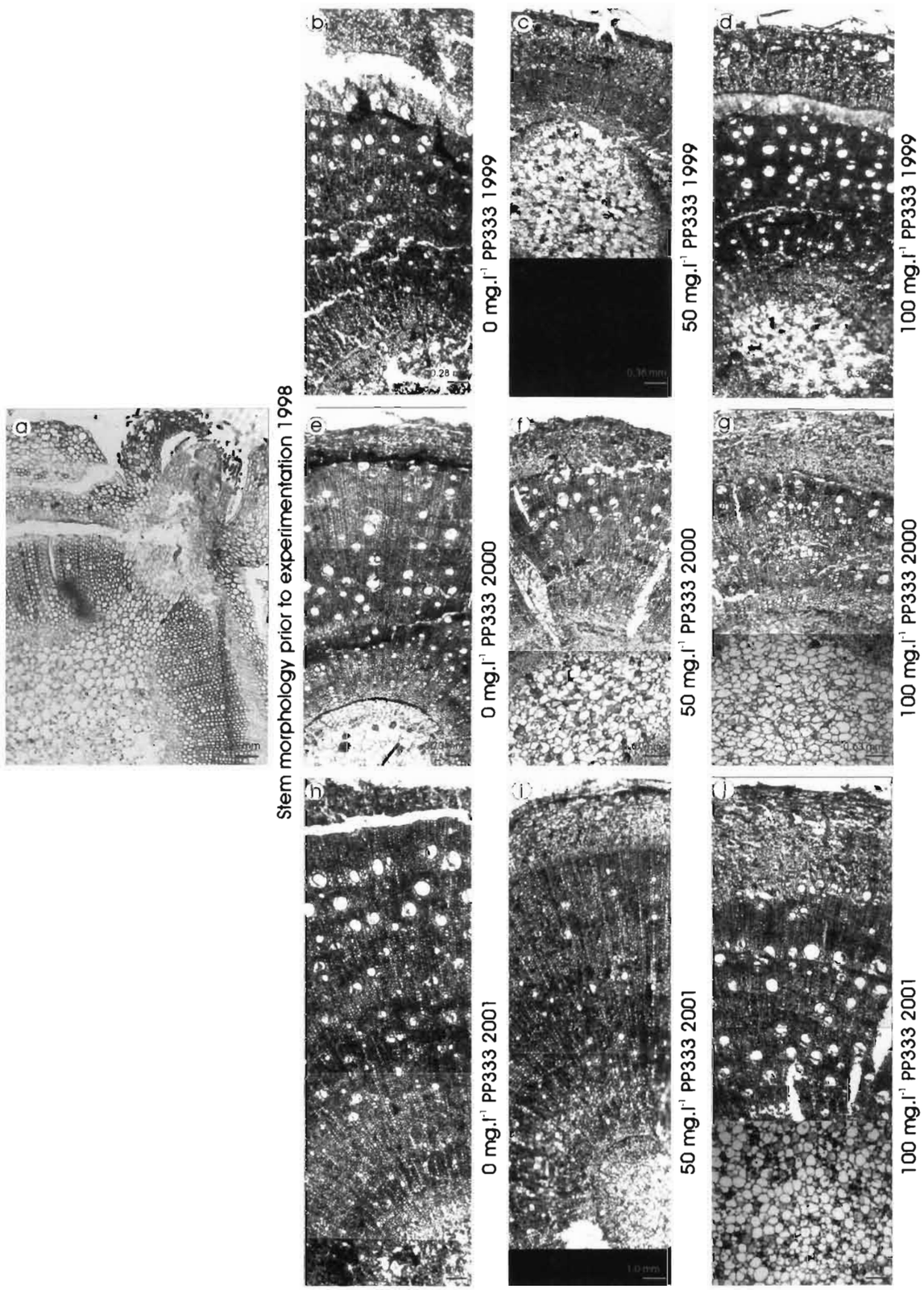

Plate 17: ***Combretum bracteosum*** **stem developmental morphology following paclobutrazol foliar spray treatments over a 36-month period**

(a) stem morphology prior to experimentation, 1998;

(b) 0 mg.ℓ^{-1} PP333 (control) 1999;

(c) 50 mg.ℓ^{-1} PP333, 1999;

(d) 100 mg.ℓ^{-1} PP333, 1999;

(e) 0 mg.ℓ^{-1} PP333 (control), 2000;

(f) 50 mg.ℓ^{-1} PP333, 2000;

(g) 100 mg.ℓ^{-1} PP333, 2000;

(h) 0 mg.ℓ^{-1} PP333 (control), 2001;

(i) 50 mg.ℓ^{-1} PP333, 2001; and

(j) 100 mg.ℓ^{-1} PP333, 2001

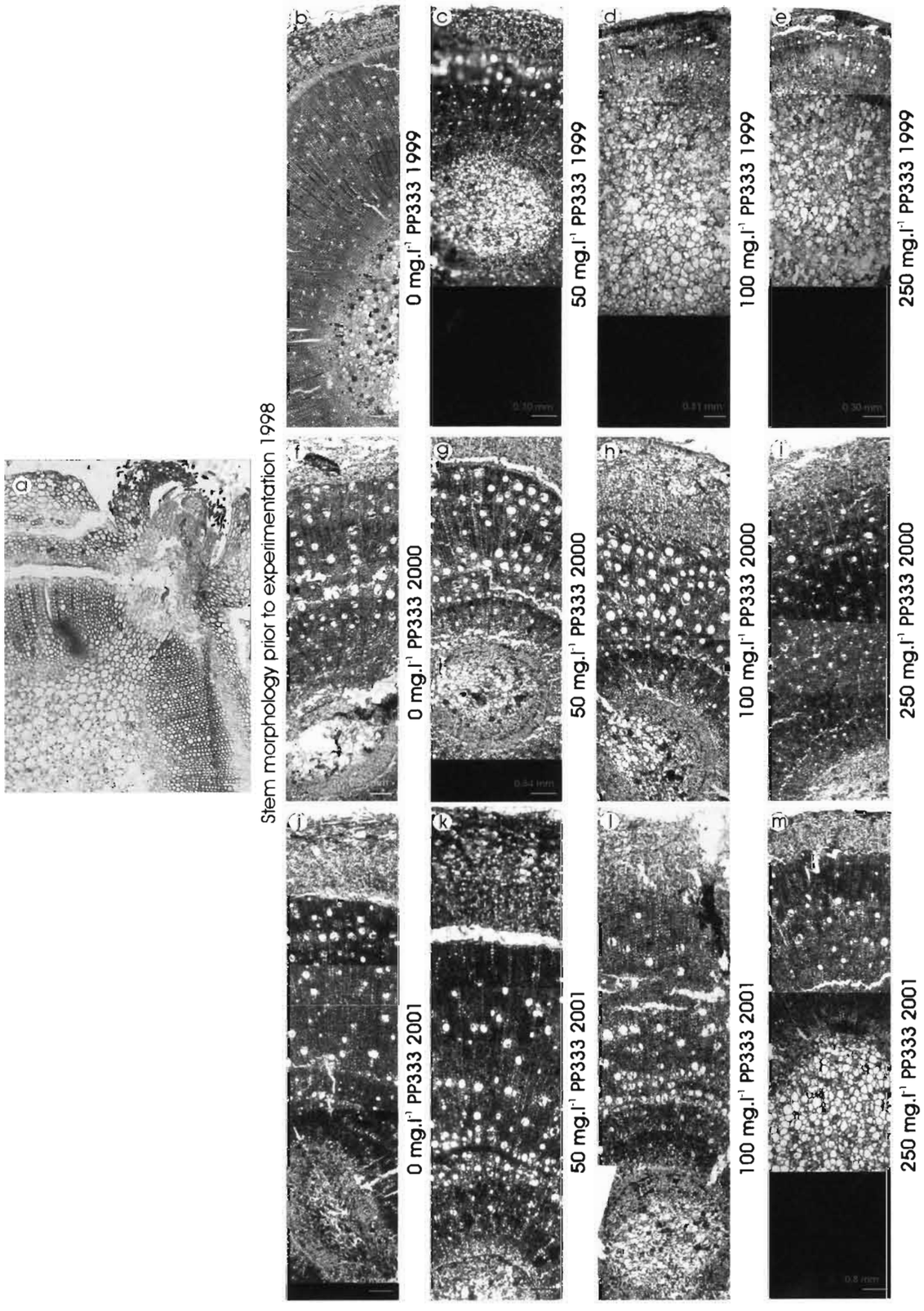

Plate 18: ***Combretum bracteosum*** **stem developmental morphology following soil drench paclobutrazol treatments over a 36-month period**

(a) stem morphology at experiment initiation, 1998;

(b) 0 mg.ℓ^{-1} PP333 (control), 1999;

(c) 50 mg.ℓ^{-1} PP333, 1999;

(d) 100 mg.ℓ^{-1} PP333, 1999;

(e) 250 mg.ℓ^{-1} PP333, 1999;

(f) 0 mg.ℓ^{-1}PP333 (control), 2000;

(g) 50 mg.ℓ^{-1} PP333, 2000;

(h) 100 mg.ℓ^{-1} PP333, 2000;

(i) 250 mg.ℓ^{-1} PP333, 2000;

(j) 0 mg.ℓ^{-1} PP333 (control), 2001;

(k) 50 mg.ℓ^{-1} PP333, 2001;

(l) 100 mg.ℓ^{-1} PP333, 2001; and

(m) 250 mg.ℓ^{-1} PP333, 2001

could result. With increasing paclobutrazol concentration, the number and diameter of the xylem vessels decreased in treated *Combretum bracteosum* plants (Plate 18). As plant height in these treatments had also decreased considerably (Figure 20 b), it can be argued that this change in xylem tissue is a result of the plant no longer requiring such a well developed water transporting system. Experimentation by MARTIN *et al.* (1987), suggests that other morphological changes that should be considered were decreases in primary and secondary xylem development and decreased annual ring thickness. These changes were however not evident in Cultar® treated *Combretum bracteosum* stem sections. PROIETTI, *et al.* (1997) added that secondary phloem development is also reduced by PP333 application, this again could not be identified clearly in *Combretum bracteosum* sections.

Obviously, when considering stem mass, values are directly related to plant height. The amount of moisture removed from the stem on drying, gives an indication of stem thickness; the thicker the stem, the greater the volume made up by plant tissue. Plates 17 and 18 illustrate that generally paclobutrazol treatment decreased stem thickness, especially in soil drenched plants. The presence of paclobutrazol appeared to be more influential at the beginning of the experiment (1999), than later, with the diameter of the stem was becoming more similar to the control. Figure 21 indicates that the control foliar spray stems had a greater fresh and dry mass than the 50 and 100 mg.ℓ^{-1} treatments. The same trend is evident from the soil drench-treated plants, although the differences recorded for the latter were more substantial. All results displayed statistical significance between treatments, over time and in the interaction between treatments and time.

In order to give a more detailed analysis, details of fresh and dry mass value differences gained at the 24-month and 36-month harvests respectively, will be discussed. This should give an indication as to whether fresh mass increases or decreases resulted from a change in water content or more importantly anatomical developmental changes. Values were recorded as follows: foliar spray control (±25 g; 33 g), 50 mg.ℓ^{-1} PP333 foliar spray (± 30 g; 18 g), 100 mg.ℓ^{-1} PP333 foliar spray (± 45 g; 20 g), control soil drench (± 47 g; 25 g), 50

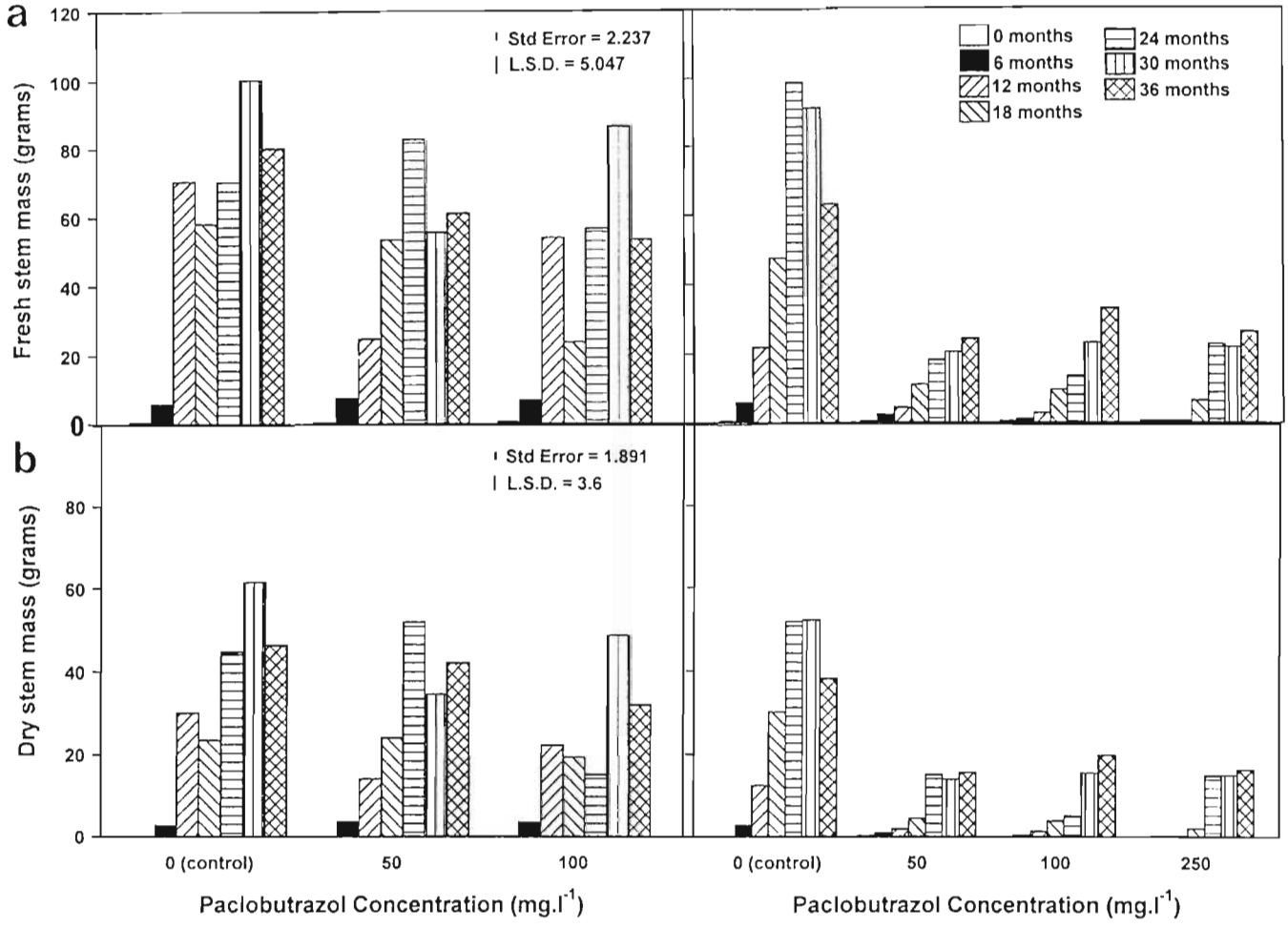

Figure 21: Effect of paclobutrazol on (a) the fresh and (b) the dry mass of *Combretum bracteosum* stems after foliar spray and soil drench treatments

mg.ℓ^{-1} PP333 (± 3 g; 8 g), 100 mg.ℓ^{-1} PP333 (± 8 g; 13 g) and 250 mg.ℓ^{-1} PP333 (± 7 g; 10 g). The difference in increase between fresh and dry mass values recorded for soil drench-treated stem, shows no specific trends across the concentration gradient. The first twelve months displayed an increase, however, for the following twenty-four months a decrease was calculated (Figure 21 b). This highlights the fact that chemical manipulation in a natural environment is never guaranteed, and the dwarfing response may fluctuate from year to year. As the quantity of stem massed for the foliar sprayed plants only decreased after twenty-four months for 50 mg.ℓ^{-1} PP333 it showed that time was also a factor in assessing effects of the dwarfing agent on the plant in question. In the case of *Combretum bracteosum*, the two different application techniques used showed different degrees of

dwarfing over time. The fact that the foliar spray treatment takes at least two years to show the desired response and the soil drench treatment displays an immediate effect reiterates the fact that the foliar spray has a delayed reaction. No conclusions could be drawn from the 100 mg.ℓ^{-1} PP333 foliar spray application in this instance as there was great unpredictable variation over the entire experimental period. The effects of triazoles on plant roots have not been studied in as much detail as their effects on shoots. Root morphology was markedly influenced by paclobutrazol treatment, especially soil drenched plants. Literature on the effects of PGR's on root growth is contradictory although reductions in volume and root diameter seem most common. Foliar spray-treated roots, were not visibly influenced by paclobutrazol treatment. As the mobility of PP333 in translocation is not great, movement of the active ingredient from the point of application (leaves) to the root was not likely, with the roots remaining unaffected (Plate 19). Reports have been made, however, that triazol-treated plants have exhibited numerous thickened, fleshy roots due to increased root diameter and decreased root length (STEFFENS *et al.,* 1985 b; SANKHLA, *et al.*, 1986; BAUSHER and YELENOSKY, 1987). Foliar spray-treated roots, as well as the control roots (foliar sprayed and soil drenched), produced a large root system which comprised mainly of very fine lateral roots coming off thick primary roots. Secondary root emergence was uniform along the entire length of the primary roots.

The effect of paclobutrazol on the appearance of the soil drenched root system was striking in that the secondary roots were no longer delicate and short, but thick and much denser. This may have been due to photosynthate partitioning since growth of the shoot (which could have stored a small amount of photosynthates) was inhibited. In comparing the volume of the control plants' root system to the treated plants' root systems, only the soil drenched plants had a severely decreased volume (Figure 22 b). All root systems appeared to remain functional (no treated plants died, or looked weak), although the appearance of root systems at higher paclobutrazol concentrations (100 and 250 mg.ℓ^{-1}) were poorly developed (Plate 20). The secondary roots were no longer evenly distributed along the length of the primary root, but tended to be aggregated just above the soil surface and along the basal region of

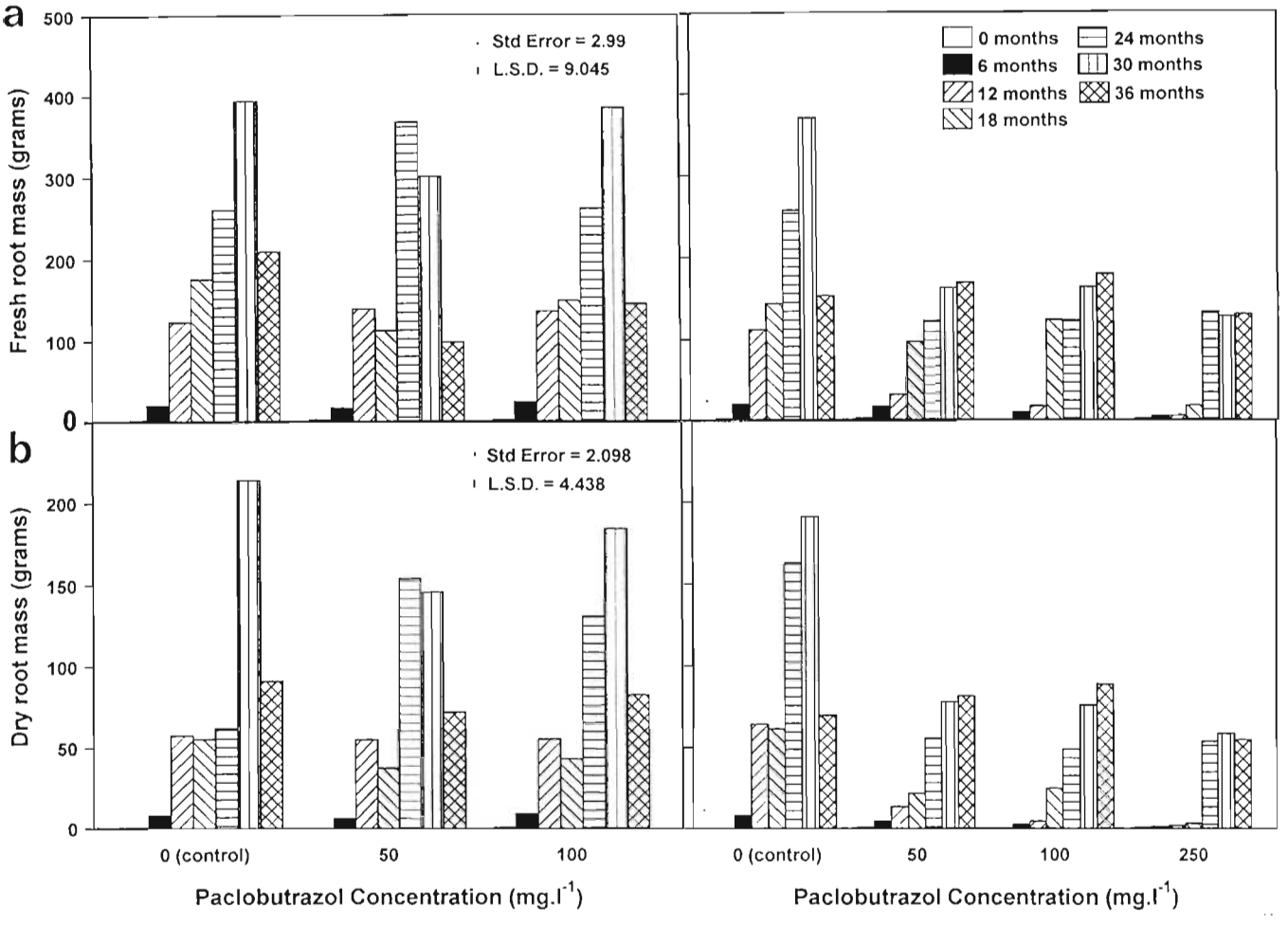

Figure 22: Effect of paclobutrazol applied as either a foliar spray or a soil drench on the (a) fresh and (b) the dry root mass of *Combretum bracteosum* plants

the primary root (perhaps where it was growing along the base of the pot). The first year after treatment, roots developing near the soil surface tended to be far longer than those further down in the soil horizon. This however, changed in subsequent years as the longer roots tended to develop near the pot base. The uncharacteristic morphological changes caused by paclobutrazol treatment appeared to be overcome by the *Combretum bracteosum* plants in time. The appearance and volume of the soil drenched root systems in 2001 looked similar to the untreated control root system, with the volume of the roots increasing and the number of swollen roots decreasing considerably (Plate 20).

There appeared to be an initial set-back in the development of the root system after the soil

drench application of PP333. Over time however, their morphology increasingly resembled the root system of the control plants. The improvement of soil drenched root system development is substantiated by the results presented in Figure 22 b, where the volume difference between the control plant root system and the treated plant root systems depict a decrease over time. The last harvest (36 months) showed that the fresh mass of the 50 mg.ℓ^{-1} and 100 mg.ℓ^{-1} was in fact greater than that of the control root system.

Although no decisive evidence to explain root thickening is apparent in *Combretum bracteosum* root sections (Plate 21 and 22), it has been suggested by WILLIAMSON and his co-workers (1986), that it could be attributed to an increase in the size of cortex parenchyma cells. This could be related to the previously mentioned storage function. From Figure 22 (a and b) water content values for foliar sprayed and soil drenched plants after 24 months were calculated. Although soil drench water content values seem low (0 mg.ℓ^{-1}, 50 g; 50 mg.ℓ^{-1}, 70 g; 100 mg.ℓ^{-1}, 15 g and 250 mg.ℓ^{-1}, 15 g), their proportion of the fresh mass value (5/11; 7/10; 3/4 and 3/4 respectively) is high. Water content in soil drenched roots increase with increasing paclobutrazol concentration, leading to an increased number of shorter, swollen roots at these concentrations. The larger water content in foliar sprayed roots was attributed to the greater root volume than their soil drenched equivalents. Only after 36 months were dwarfing effects in foliar sprayed root systems evident, with fresh masses decreasing to almost one-third their mass one year previously (Figure 22 a).

The first visible effect of PP333 application was the darkening of the leaves. This phenomenon, although visible from four weeks after treatment, was not supported by the chlorophyll extraction analysis after six months. Following trends recorded in the literature (SANKHLA, *et al.*, 1985; WANG, *et al.*, 1985; TYMOSZUK and MIKA, 1986), soil drenched leaves appeared to get smaller and darker with increasing PP333 concentration (Figure 23 b and Figure 26 b). This was especially true for leaves that developed after paclobutrazol application (Plate 16 c and d). After six months, the chlorophyll content of soil drench treated plants were all lower than that of the control plant. Although levels of chlorophyll measured in the leaves moved closer to those of the control with increasing

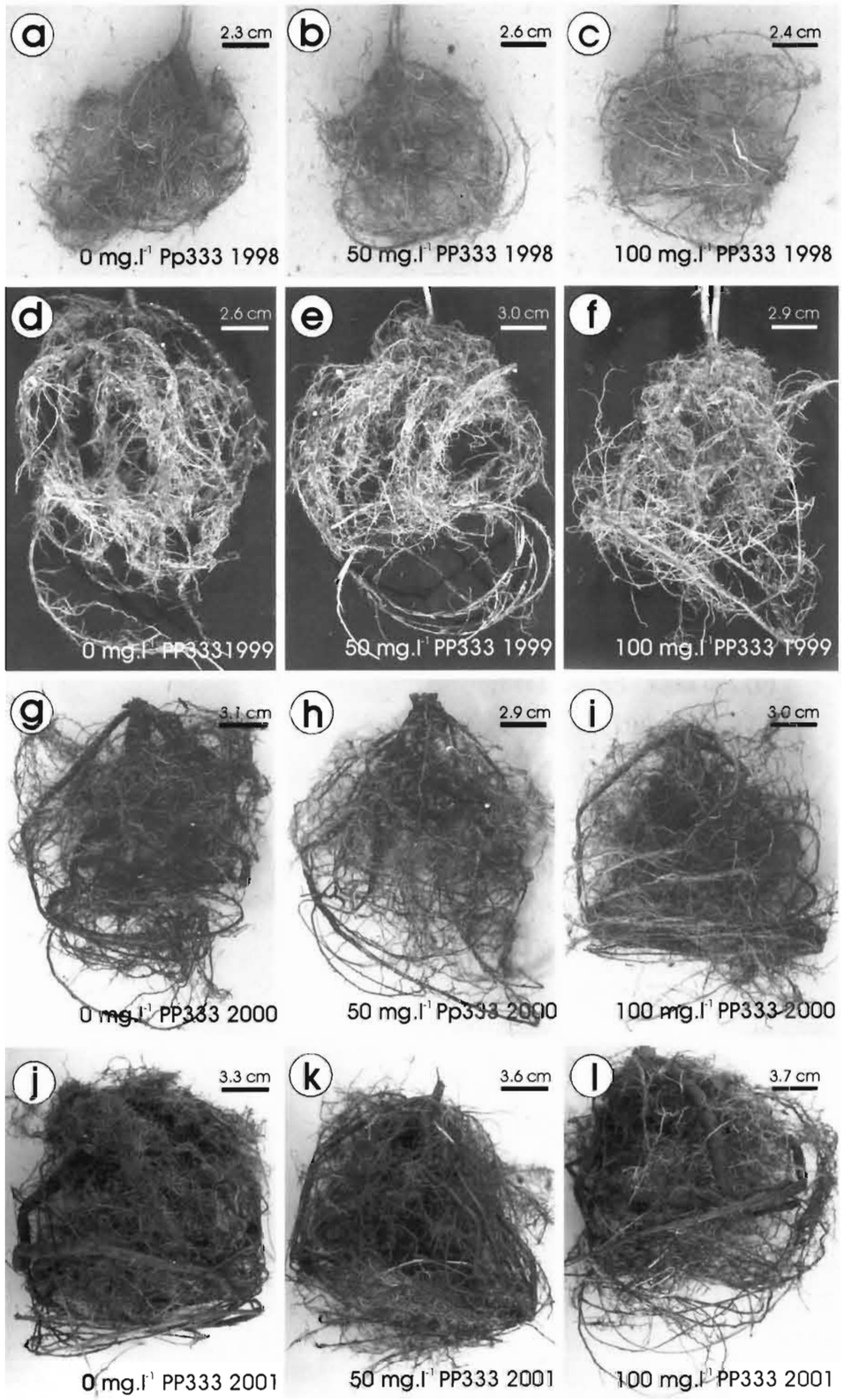

Plate 19: *Combretum bracteosum* external root morphology following paclobutrazol foliar spray treatments over a 36-month period

(a) 0 mg.ℓ^{-1} PP333 (control), 1998;

(b) 50 mg.ℓ^{-1} PP333, 1998;

(c) 100 mg.ℓ^{-1} PP333, 1998;

(d) 0 mg.ℓ^{-1} PP333 (control) 1999;

(e) 50 mg.ℓ^{-1} PP333, 1999;

(f) 100 mg.ℓ^{-1} PP333, 1999;

(g) 0 mg.ℓ^{-1} PP333 (control), 2000;

(h) 50 mg.ℓ^{-1} PP333, 2000;

(i) 100 mg.ℓ^{-1} PP333, 2000;

(j) 0 mg.ℓ^{-1} PP333 (control), 2001;

(k) 50 mg.ℓ^{-1} PP333, 2001; and

(l) 100 mg.ℓ^{-1} PP333, 2001

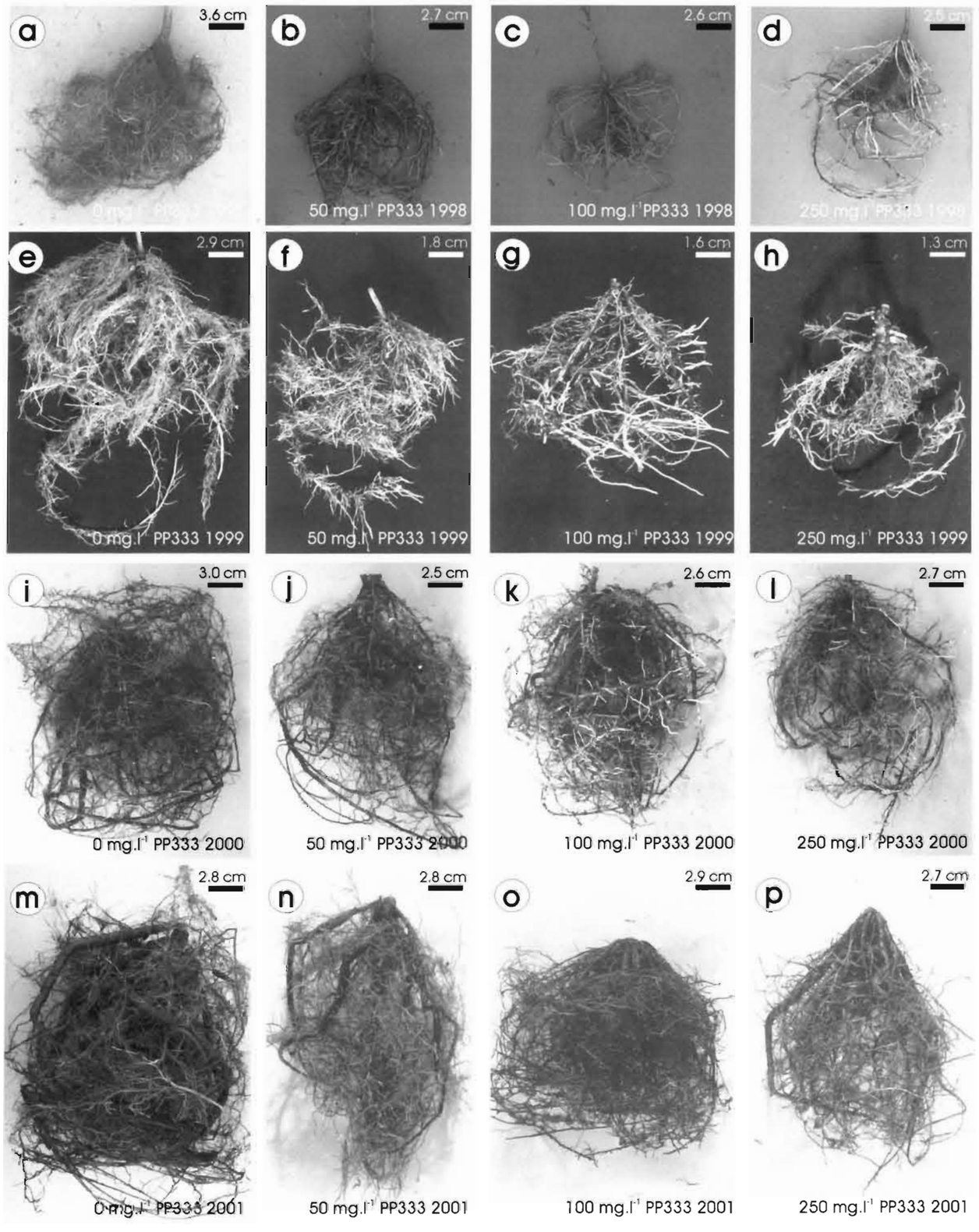

Plate 20: ***Combretum bracteosum*** **external root morphology following paclobutrazol soil drench treatments over a 36-month period**

(a) 0 mg.ℓ^{-1} PP333 (control), 1998;

(b) 50 mg.ℓ^{-1} PP333, 1998;

(c) 100 mg.ℓ^{-1}PP333, 1998;

(d) 250 mg.ℓ^{-1} PP333, 1998;

(e) 0 mg.ℓ^{-1} PP333 (control), 1999;

(f) 50 mg.ℓ^{-1} PP333, 1999;

(g) 100 mg.ℓ^{-1} PP333, 1999;

(h) 250 mg.ℓ^{-1} PP333, 1999;

(i) 0 mg.ℓ^{-1}PP333 (control), 2000;

(j) 50 mg.ℓ^{-1} PP333, 2000;

(k) 100 mg.ℓ^{-1} PP333, 2000;

(l) 250 mg.ℓ^{-1} PP333, 2000;

(m) 0 mg.ℓ^{-1} PP333 (control), 2001;

(n) 50 mg.ℓ^{-1} PP333, 2001;

(o) 100 mg.ℓ^{-1} PP333, 2001; and

(p) 250 mg.ℓ^{-1} PP333, 2001

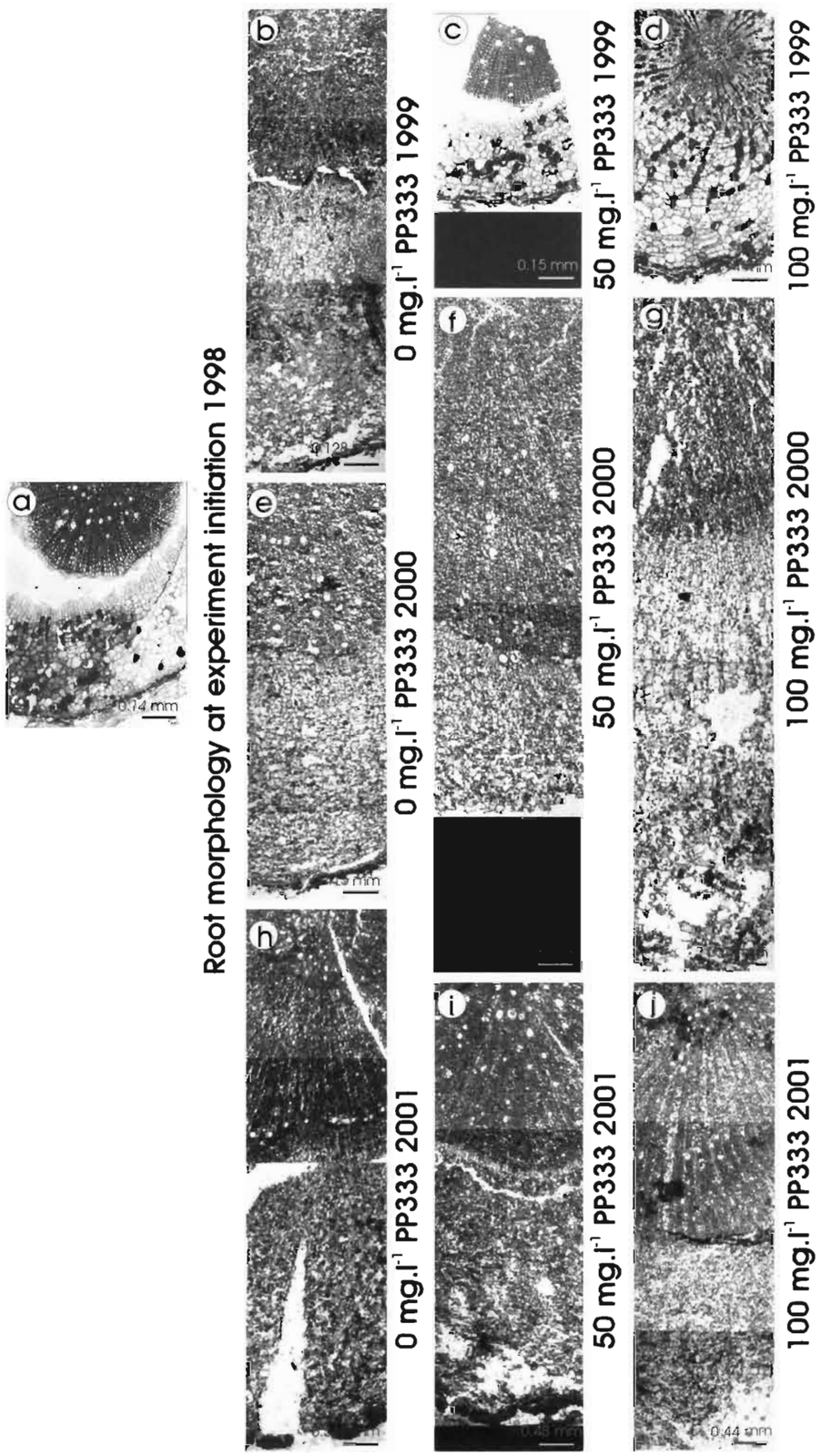

Plate 21: ***Combretum bracteosum*** **root developmental morphology following paclobutrazol foliar spray treatments over a 36-month period**

(a) root morphology prior to experimentation, 1998;

(b) 0 mg.ℓ^{-1} PP333 (control) 1999;

(c) 50 mg.ℓ^{-1} PP333, 1999;

(d) 100 mg.ℓ^{-1} PP333, 1999;

(e) 0 mg.ℓ^{-1} PP333 (control), 2000;

(f) 50 mg.ℓ^{-1} PP333, 2000;

(g) 100 mg.ℓ^{-1} PP333, 2000;

(h) 0 mg.ℓ^{-1} PP333 (control), 2001;

(i) 50 mg.ℓ^{-1} PP333, 2001; and

(j) 100 mg.ℓ^{-1} PP333, 2001

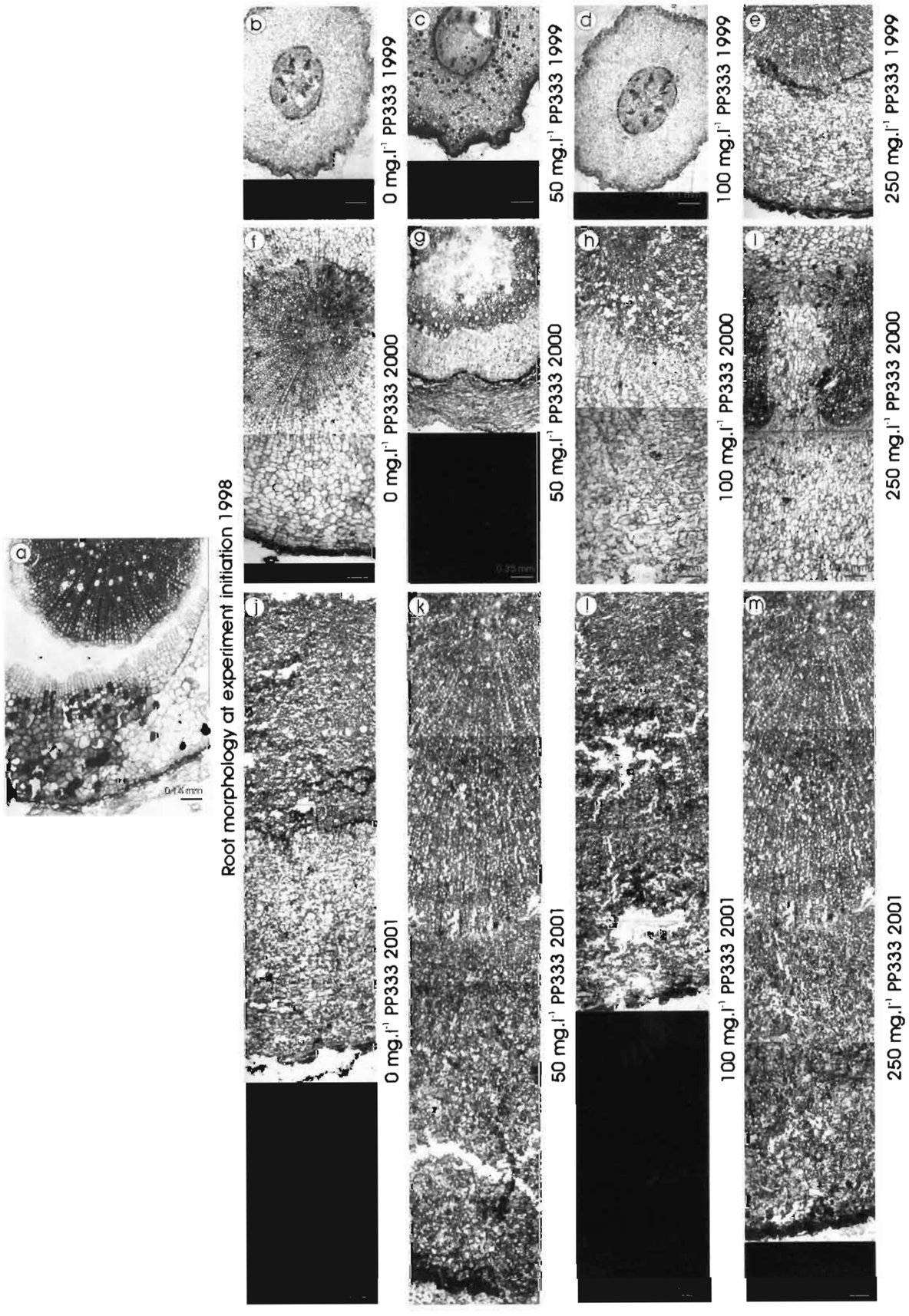

Plate 22: ***Combretum bracteosum*** **root developmental morphology following soil drench paclobutrazol treatments over a 36-month period**

(a) root morphology at experiment initiation, 1998;

(b) 0 mg.ℓ^{-1} PP333 (control), 1999;

(c) 50 mg.ℓ^{-1} PP333, 1999;

(d) 100 mg.ℓ^{-1} PP333, 1999;

(e) 250 mg.ℓ^{-1} PP333, 1999;

(f) 0 mg.ℓ^{-1}PP333 (control), 2000;

(g) 50 mg.ℓ^{-1} PP333, 2000;

(h) 100 mg.ℓ^{-1} PP333, 2000;

(i) 250 mg.ℓ^{-1} PP333, 2000;

(j) 0 mg.ℓ^{-1} PP333 (control), 2001;

(k) 50 mg.ℓ^{-1} PP333, 2001;

(l) 100 mg.ℓ^{-1} PP333, 2001; and

(m) 250 mg.ℓ^{-1} PP333, 2001

paclobutrazol concentrations. It is also interesting to note that the leaves treated with a 50 mg.ℓ^{-1} paclobutrazol soil drench showed a decrease in chlorophyll content since the beginning of experimentation (approximately 5 mg.ℓ^{-1}). All other soil drench treatments showed an increase in chlorophyll levels over the same six month period. One year after initial treatment (September 1999), the levels of chlorophyll in the leaves were still increasing in the soil drench control plants (Figure 23 b). The darker colouring of the leaves (Plate 16 a - d) in this case were substantiated by chlorophyll extraction values, with the amount of chlorophyll per unit volume increasing with increasing paclobutrazol concentration. With the exception of the 250 mg.ℓ^{-1} soil drench treated leaves, chlorophyll content differences noted between the control and the drench treated plants' leaves during 1999 (Figure 23 b and Plate 16 e - f) were relatively small.

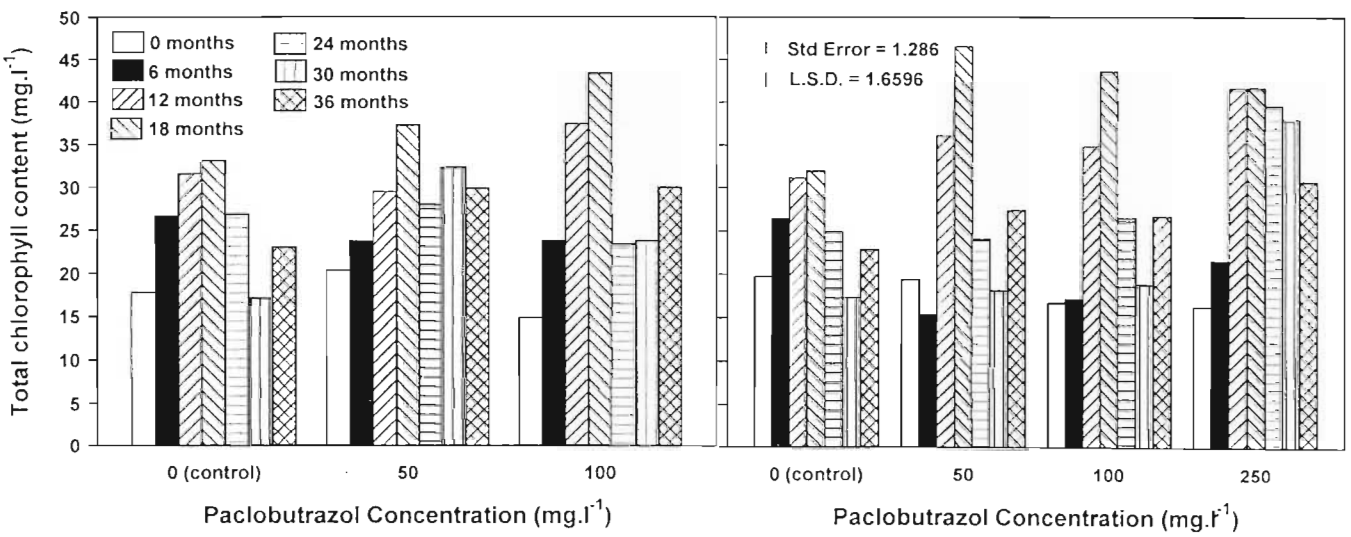

Figure 23: Total chlorophyll content of *Combretum bracteosum* leaves treated with paclobutrazol (a) foliar spray and (b) soil drench

The deeper green appearance of treated leaves is not clearly supported by total chlorophyll content values. Examination of chlorophyll a (blue-green colouration) and chlorophyll b (yellow-green colouration) levels, independently indicates that chlorophyll a values increase

with increasing PP333 concentration. Although the total chlorophyll content showed a statistically significant difference with increasing paclobutrazol concentration, it was probably the chlorophyll a alone that led to the visible enhancement of green colouration in the soil drench treated leaves.

A general trend seen in all *Combretum bracteosum* plants treated with a foliar spray of paclobutrazol, irrespective of concentration (control, 50, 100 or 250 mg.ℓ^{-1}) was the consistent increase in chlorophyll content for the first two years, subsequent to that all chlorophyll levels dipped to various lows before regaining some of their losses. The chlorophyll content values recorded for soil drenched plants, were less consistent and predicable. The amount of chlorophyll recorded for soil drenched plants was more than the foliar spray-treated plants at the same concentration. Paclobutrazol applied at 50 mg.ℓ^{-1} and 100 mg.ℓ^{-1} showed the same response trends as foliar sprayed plants, whereas at 250 mg.ℓ^{-1} PP333 the initial increase was followed by a steady decrease, with no intermittent fluctuations (Figure 23 a).

It is important to bear in mind that seasonal variation also plays a part in leaf chlorophyll content and also has to be considered when analysing data, especially as harvesting was undertaken at the end of September and the end of March. The reason these times were chosen, although relatively close to a seasonal change is that during this time climatic conditions are moderate, and plants were assumed to be under minimal stress (water, heat, nutrient, etc.). Enough time was allowed in spring to ensure full leaf expansion and other vegetative growth, as well as strict control being taken prior to autumn to ensure the elimination of possible senescing plants. Results obtained were assumed to be representative of healthy plants under minimal stress for an experiment under natural conditions. Results do not reflect any unusual seasonal effects.

In analysing chlorophyll content results statistically over the thirty-six month period, each month was significant from the others. The two treatment types (foliar spray vs. soil drench)

were also significantly different, as was the interaction between treatment type and time in all instances.

The discussion as to why leaves from different paclobutrazol treatments manifest different shades of green should now be addressed from a morphological perspective. Have treatments resulted in an increased chloroplast number within each cell, or has the cell number (each containing their original number of chloroplasts) increased, resulting in a thickening of the leaves? In order to answer this, sections of leaf material have been examined under the light microscope. Although average leaf chlorophyll contents for each PP333 concentration over the entire experimental period (1998 - 2001) show that neither foliar spray nor soil drench treatments differ significantly (statistically), other factors that could be contributing to the intensified green colouring (clearly visible to the observer) should not be overlooked. The thickness of leaf laminae of grasses, sugar beets (JAGGARD *et al.*, 1982; DALZIEL and LAWRENCE, 1984), peaches (EARLY and MARTIN, 1989) and pecans (WOOD, 1984) among others, were increased by paclobutrazol treatment. The larger surface area of mesophyll cells in the thicker leaves allowed for greater exposure of the intercellular spaces, increasing the opportunity for CO_2 exchange to the sites of photosynthesis (TREHARNE, 1982).

A visual inspection of the soil drench treated leaves revealed a thickening of the lamina as well as an intensified green pigmentation. Indications were that this was not due to an increase in the thickness of the upper and lower epidermis, or additional layers in the spongy parenchyma, but a decrease in the volume of the cells' intercellular spaces with increasing PP333 concentration (Plate 24). With the cells being packed more closely to one another, an intensified green pigmentation resulted (Plate 18). Consistent with the findings of PROIETTI, *et al.* (1997), it appears that foliar spray treatments did not significantly influence leaf development (Plate 23). The vascular system development of the leaf remained unaffected by both treatment techniques.

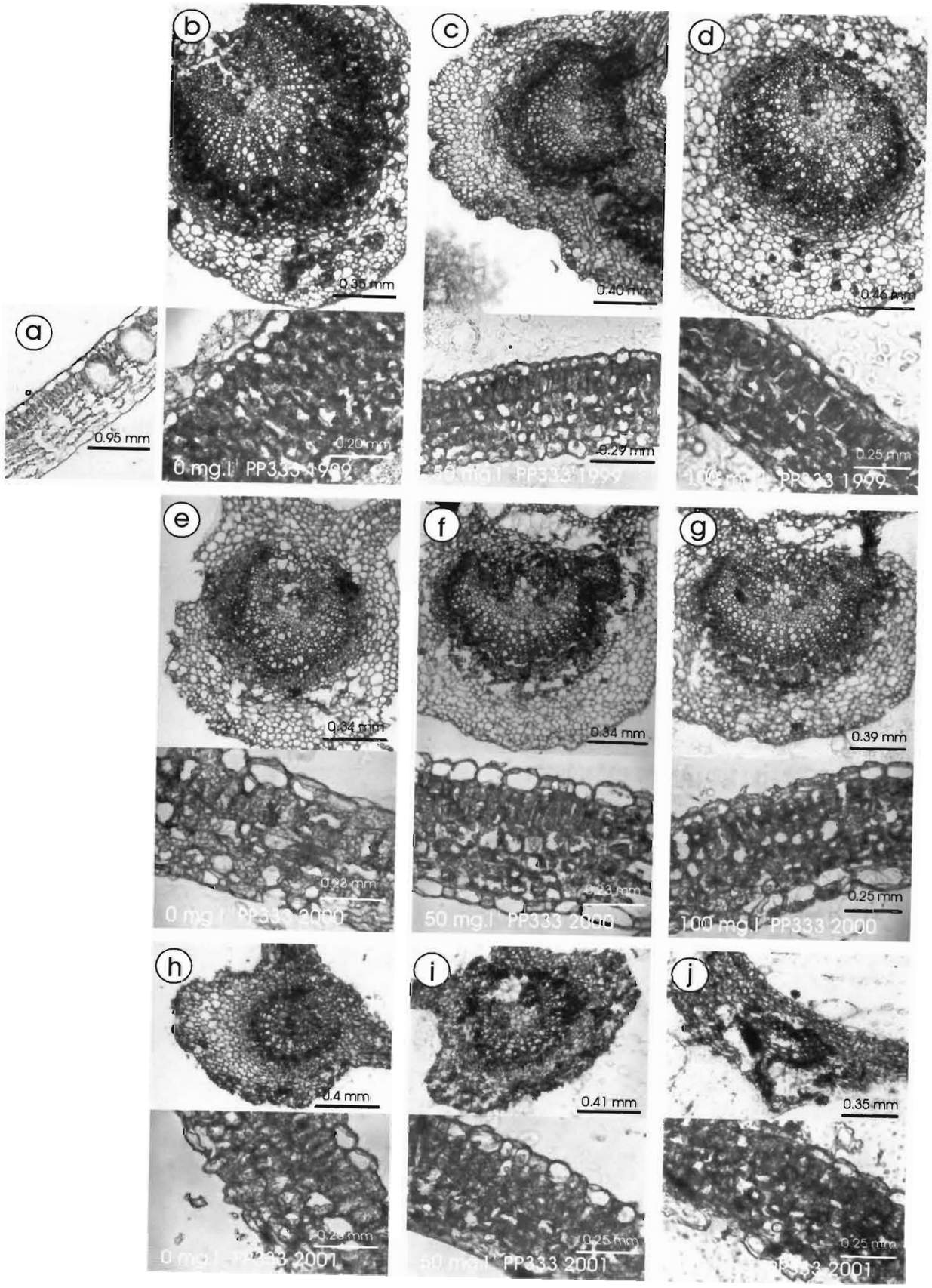

Plate 23: ***Combretum bracteosum*** **leaf sections illustrating morphological changes brought about by paclobutrazol foliar spray treatments**

(a) leaf morphology prior to experimentation, 1998;

(b) 0 mg.ℓ^{-1} PP333 (control) 1999;

(c) 50 mg.ℓ^{-1} PP333, 1999;

(d) 100 mg.ℓ^{-1} PP333, 1999;

(e) 0 mg.ℓ^{-1} PP333 (control), 2000;

(f) 50 mg.ℓ^{-1} PP333, 2000;

(g) 100 mg.ℓ^{-1} PP333, 2000;

(h) 0 mg.ℓ^{-1} PP333 (control), 2001;

(i) 50 mg.ℓ^{-1} PP333, 2001; and

(j) 100 mg.ℓ^{-1} PP333, 2001

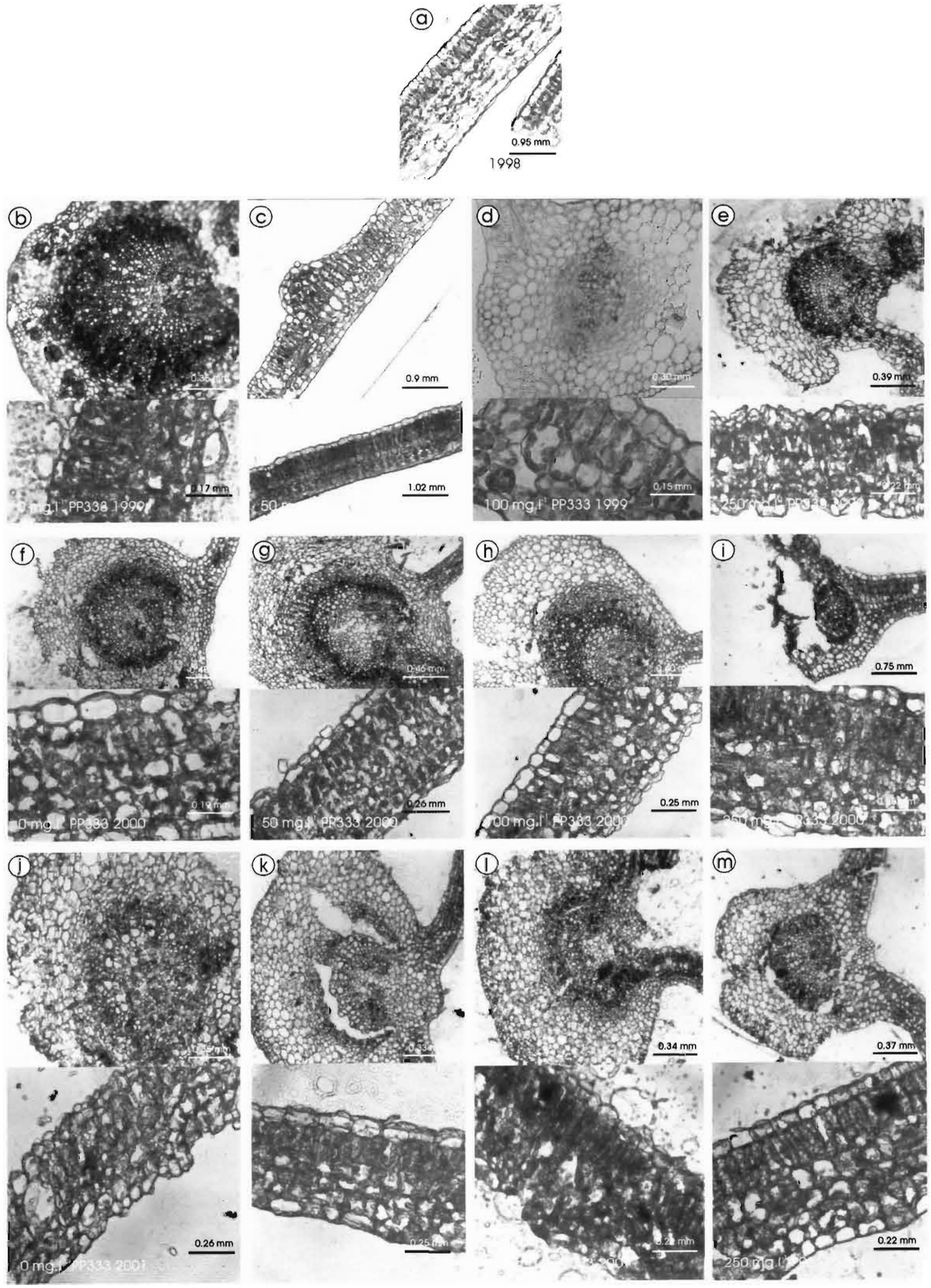

Plate 24: ***Combretum bracteosum*** **leaf sections illustrating morphological changes brought about by paclobutrazol soil drench treatments**

(a) leaf morphology at experiment initiation, 1998;

(b) 0 mg.ℓ^{-1} PP333 (control), 1999;

(c) 50 mg.ℓ^{-1} PP333, 1999;

(d) 100 mg.ℓ^{-1} PP333, 1999;

(e) 250 mg.ℓ^{-1} PP333, 1999;

(f) 0 mg.ℓ^{-1}PP333 (control), 2000;

(g) 50 mg.ℓ^{-1} PP333, 2000;

(h) 100 mg.ℓ^{-1} PP333, 2000;

(i) 250 mg.ℓ^{-1} PP333, 2000;

(j) 0 mg.ℓ^{-1} PP333 (control), 2001;

(k) 50 mg.ℓ^{-1} PP333, 2001;

(l) 100 mg.ℓ^{-1} PP333, 2001; and

(m) 250 mg.ℓ^{-1} PP333, 2001

In comparing the differences between fresh and dry mass for the harvested *Combretum bracteosum* plants (Figure 24 a and b), the expected increased mass difference with increasing PP333 concentration was only evident in the foliar spray-treated plants. Changes in mass differences across treatments are represented by values given for 24 month and 36 month harvested leaf masses respectively, foliar spray control (± 32 g; 14 g), 50 mg.ℓ^{-1} foliar spray (± 37 g; 16 g), 100 mg.ℓ^{-1} foliar spray (± 52 g; 12 g), control soil drench (± 52 g; 16 g), 50 mg.ℓ^{-1} soil drench (± 12 g; 11 g), 100 mg.ℓ^{-1} soil drench (± 10 g; 16 g) and 250 mg.ℓ^{-1} soil drench (± 20 g; 13 g). Foliar spray-treated leaf masses tended to follow similar trends set by the control (untreated) plants, with peaks and dips in mass recorded at the same intervals. The increased leaf mass with increasing PP333 concentrations was only evident within the first three years, after which very little difference between treatments was found (Figure 24 a). A possible explanation for these trends could be that there is an increase in photosynthesis in foliar sprayed plants, resulting in more photosynthates being produced per leaf. Experiments by BLANCO *et al.* (1997) demonstrated that PP333 modifies the efficiency of photosystems I and II.

To begin with, soil drench-treated plants however displayed a decrease in leaf mass difference with increasing PP333 concentration (Figure 24 b) although later in the experiment the higher concentrations induced an increase in leaf mass, indicating the reduction in water content within these treated tissues. The higher dry mass values indicates that the retardant had enhanced dry matter yield per leaf, which is consistent with the fact that a few more parenchyma cells are found in the leaf blade (Plate 18). Results obtained for leaf mass analysis (between treatments, over time and the interaction between treatments and time) were all statistically significant from one another.

The values recorded for the fresh and dry leaf masses are directly related to the number of leaves collected and the total leaf area recorded from each harvested *Combretum bracteosum* plant. On average, the number of leaves recorded for each plant (Figure 25) increased in the foliar spray treatments, and decreased in the soil drench treatments, with increasing PGR

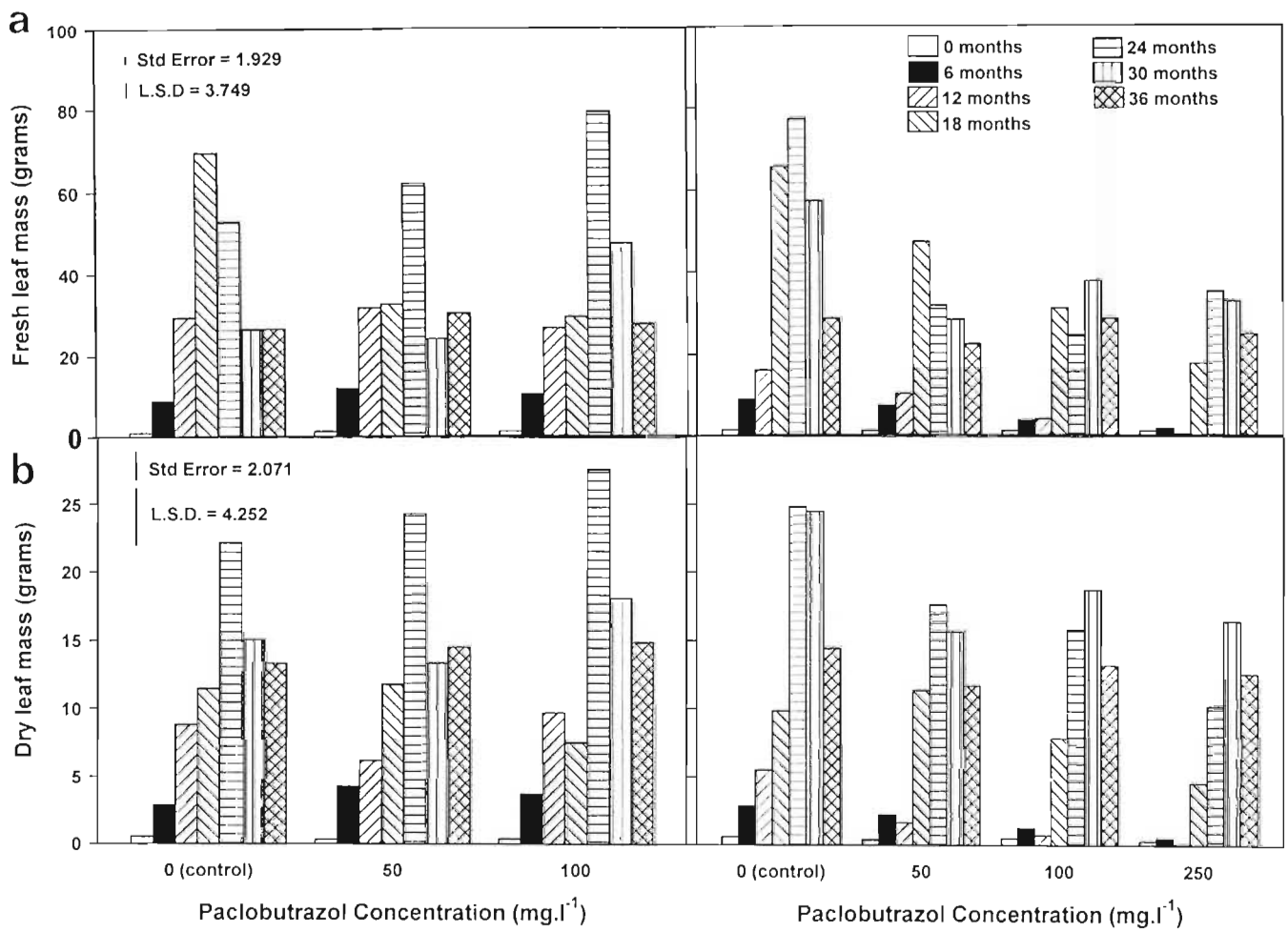

Figure 24: Effect of paclobutrazol treatment in the (a) fresh and (b) the dry mass of *Combretum bracteosum* leaves following either a foliar spray or a soil drench treatment

concentration. On closer analysis of this trend, the number of *Combretum bracteosum* leaves collected within 1998 and 1999 increased at each harvest interval, whereas in later years (2000 and 2001) the leaf numbers dropped.

In drawing comparisons between treated plants and their respective control plants, the foliar spray tended to cause great variation in leaf numbers within the year, whereas the soil drench-treated plants tended to maintain a more uniform leaf number throughout the year. Figure 25 (a) illustrates that leaf number on the control plants increased slowly and steadily.

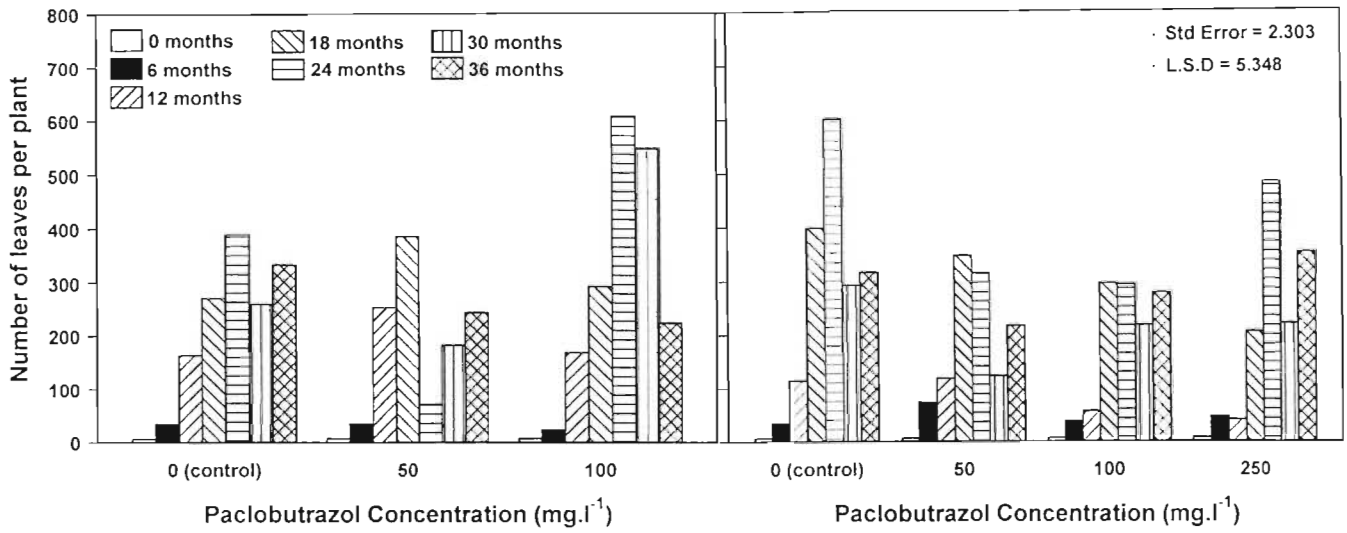

Figure 25: Change in leaf number on *Combretum bracteosum* plants after a (a) foliar spray or (b) soil drench paclobutrazol treatment

The 50 mg.ℓ^{-1} PP333-treated plants showed a reduction in vegetative growth, as during the year 2000 the number of leaves recorded on the plants had diminished, leaving the appearance of the treated plants bare and undesirable. The applied dwarfing agent only induced a suitable habit for the *Combretum bracteosum* plant three years after the initial application (in 2001), as not only was plant height reduced, but the number of leaves per plant were also increased (Plate 15). The previously untidy, shabby looking *Combretum bracteosum* plant therefore appeared more compact and attractive. Plants treated with 100 mg.ℓ^{-1} showed even more variation in leaf number changes over time, with the increase and decrease in leaf number being rapid and large. The 100 mg.ℓ^{-1} PP333-treated plants, however again ended up being bushy, dwarfed plants by the year 2001. The soil drench treatment is thought to have had a stabilizing effect on leaf number variation over time, with the differences in leaf number decreasing with increasing PP333 concentration. Inconsistent with this trend, however, was the variation in leaf number in the 250 mg.ℓ^{-1} PP333-treated plants. This is thought to be a consequence of the foliar spray application (1999) in an attempt to increase leaf area (Figure 24 b). The number of leaves that developed on soil

drenched plants added to their suitability as attractive dwarfed *Combretum bracteosum* plants (Plate 16). A significant statistical difference was recorded for leaf numbers per plant between each six-month interval and for the interaction between treatment type and time. There was however no statistical significance between the two treatment types used.

Leaf area was effected considerably by paclobutrazol treatments. Figure 26 (a) highlights the fact that foliar spray-treated plants displayed a leaf area greater than both the control and the soil drench-treated leaves. Foliar sprayed plants, during the years 1998, 1999 and 2000, recorded leaves with an increasing leaf area at each harvest. It however appeared to begin decreasing during 2001. The smaller leaf area (year 2001) was more consistent with expected paclobutrazol effects and again substantiates the assumption that the foliar spray as a dwarfing agent only becomes effective in its third year.

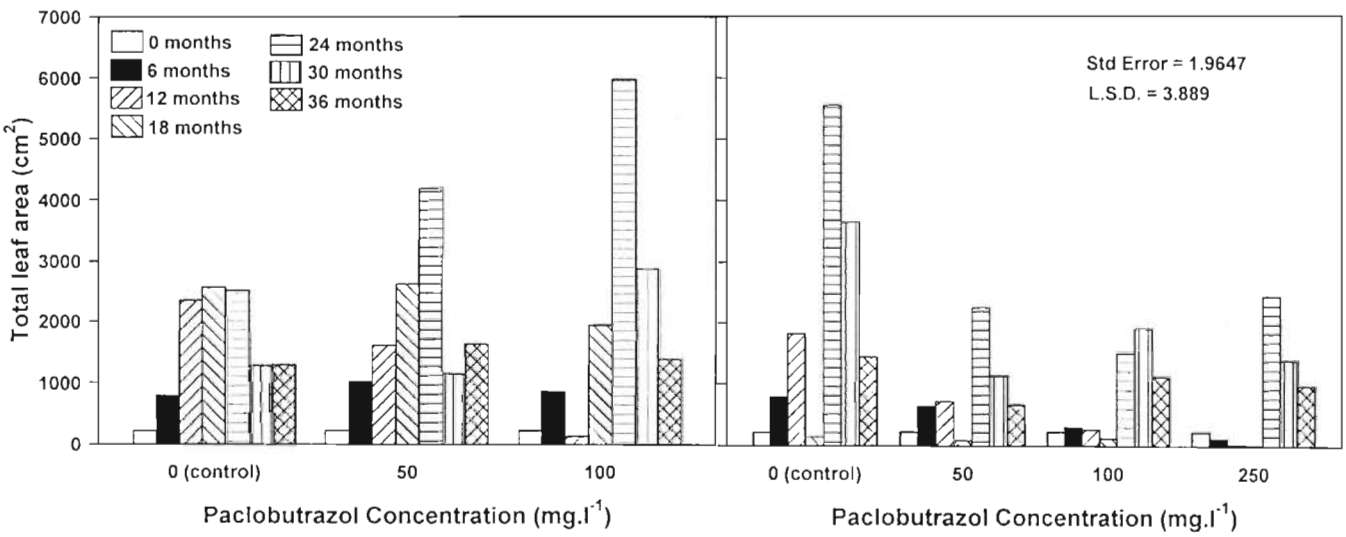

Figure 26: Effect of different PP333 treatments and concentrations on the leaf area of *Combretum bracteosum* plants

The soil drenched plants were severely effected by paclobutrazol treatments, especially at higher concentrations (Plate 16 h). The minute, curled leaves were undesirable and was one of the primary reasons for excluding 250 mg.ℓ^{-1} PP333 soil drench treatments from recommendation for commercial use for *Combretum bracteosum* plants. After a 100 mg.ℓ^{-1} PP333 foliar spray replaced the 250 mg.ℓ^{-1} PP333 soil drench application in 2000, the leaf

area increased to a more desirable size. Figure 26 (b) however indicates that this increase appears to be short-lived and the leaf area recorded in 2001 decreased again. Together with the decreased leaf area caused by the soil drench PGR application, the leaf margins curled severely (YOUNG, 1983; DAVIS, 1987), with the degree of curling increasing with increasing paclobutrazol concentration (Plate 16). However, the older the plants became, the less noticeable the curling was.

A statistical significance was recorded for all results obtained concerning *Combretum bracteosum* leaf area, namely differences between treatment types, changes over time and differences in the interaction between treatment type and time.

4.4 Conclusions

The triazoles are highly active plant growth regulators which hold considerable promise for a number of horticultural applications, including the dwarfing of *Combretum bracteosum*. The ability to consistently regulate growth at low dosages, its ease of application and its general lack of phytotoxicity, are major advantages that paclobutrazol has over many other growth retardants.

Although at the end of the experimental period, all paclobutrazol treatments had resulted in dwarfed plants, a 50 mg.ℓ^{-1} soil drench treatment is recommended as the most suitable concentration and application method. Spraying *Combretum bracteosum* leaves in order to induce a retarded growth effect, only produced the desired effect approximately two years after initial treatment. This slow response rate would not be viable in a commercial set-up, where the turn-over of plants is ideally only a few months. Concentrations of paclobutrazol higher than 50 mg.ℓ^{-1} (100 mg.ℓ^{-1} and 250 mg.ℓ^{-1}) applied as a soil drench, although inducing dwarfing effects within a few weeks, were responsible for some uncharacteristic morphological developments, which again would be undesirable from a sales point of view. People wanting to buy plants, even if they are unaware of the unique dwarfed appearance of the *C. bracteosum* plants, would not be interested in buying if the specimens had no

lateral branches and a few minute, curled-up leaves.

The compact, bushy habit resulting from the 50 mg.ℓ^{-1} PP333 treatment, is ideal for use as a pot-plant or a small garden plant. No undesired characteristics were associated with dwarfed plants treated with paclobutrazol at a concentration of 50 mg.ℓ^{-1}, or with the soil drench method of application.

Chapter 6

General Conclusion

In order to maximize the beauty of South Africa's floral diversity, plant propagators have begun exploiting the rich array of flowering plants found within the different biomes of our country. This trend is now also followed by a large number of avid gardeners, professionals and amateurs alike. With the move away from the use of exotic plants, nurserymen are now having to produce stocks of indigenous flora in large numbers to meet the increasing demand by urban gardeners. Maintaining strict conservation ethics, but still attempting to promote the use of indigenous plants, rapid, easy and cost effective methods of propagating these plants need to be established.

Combretum bracteosum is one such indigenous plant, the aesthetic appeal thereof exhibits great potential for ornamentation, especially when flowering. In facilitating the more successful development of indigenous plants into small gardens or even into pots, this thesis set out to investigate, analyse and determine quick and easy methods of propagating *Combretum bracteosum*. Various aspects of propagation were considered namely seed germination, propagation from cuttings, and micropropagation (which included seed germination *in vitro*, axillary bud elongation and meristem culture). With the exception of micropropagation, little specialised skill and training would be required prior to carrying out the relevant procedures and protocols, ensuring their accessibility to most plant propagators. The successful results obtained in propagating *Combretum bracteosum* using simple propagation techniques such as germinating the seed or rooting cuttings enables propagators with different levels of experience and expertise to repeat the experimental procedures successfully and with ease.

Propagation by seed germination yielded less than optimal results from a commercial perspective, however propagators not economically dependant on high germination rates

such as propagators cultivating the seed for private purposes, could generate healthy plantlets within a few weeks. Highly problematic in using *Combretum bracteosum* seed as a seedling stock source is the fluctuating levels of seed availability from year to year. The hard pericarp surrounding the embryo also appeared to impose a mechanical hindrance to the emerging radicle and developing roots, resulting in the time consuming preparation of pericarp scarification being essential. Further factors that influenced successful propagation through seed germination were temperature and light (which enhanced germination at optimal levels), and the recalcitrant characteristics of the seed (which hampered germination).

A more viable and simpler alternative to seed germination, was propagation by stem cuttings. The technique of striking cuttings provided promising results for the successful cultivation of *Combretum bracteosum*. Root induction on the cuttings was not only influenced by hormonal treatments, but was highly dependant on optimal extrinsic conditions being met. In encouraging propagation of indigenous plants for gardening purposes, it is important to bear in mind that hormonal supplements used in a laboratory set-up may not be available to nurserymen and private gardeners. The use of the commercially available Kelpak as an exogenous hormone supplement for rapid root development, was therefore suitable in achieving the propagation aims, as accessibility of all the required materials for striking *Combretum bracteosum* cuttings was almost guaranteed. Subsequent to striking the cuttings', time was still required by the propagator as close attention needed to be paid to the cuttings water and nutrient availability as well as their surrounding atmospheric temperature. Once the development of a root system was initiated however, the plantlets became more independent in that they no longer required nutrient supplementation and they were able to endure water and temperature fluctuations more readily.

Generating plantlets using micropropagation techniques requires skilled labour, expensive equipment and chemicals, time and patience. Success in generating *Combretum*

bracteosum plantlets was obtained by germinating the seed *in vitro* as well as stimulating axillary shoot elongation from nodal explants. These two methods are probably the easiest and quickest means of propagation *in vitro*, so it was not essential that more complex vegetative reproduction alternatives be investigated. It was the high contamination rates during some periods of the year however that led to the experimentation with meristem explants. Rapid cell division within the meristematic zone is thought to out-compete, and result in the elimination of viruses and other contaminants within the explant even before placing them into culture. Although the contamination problem was reduced, plantlet regeneration from meristem explants was unsuccessful. This was probably due to the inability to determine the exact hormone or hormone combination that could initiate the elongation of the meristematic zone.

With a successful protocol for the elongation of axillary buds having been developed, already sterile explants from seed germinated *in vitro,* provided a large stock base from which further multiplication of *Combretum bracteosum* plants could be continued. Although limited to institutions with tissue culture facilities, this rapid and reliable protocol provides great possibilities for the economic viability of this application, and is perhaps the best method of propagating *Combretum bracteosum* for commercial purposes. The use of already sterile explants (from seed germinated *in vitro*), also ensured that this procedure was less labour intensive than the alternative method of collecting nodal explants from the parent plants growing in their natural environment and then having to sterilize them. Fungal and bacterial contamination was highly problematic with the latter mentioned explants, where at times up to 60% were lost to contamination. Those explants being able to overcome the initial decontamination steps, were however, equally successful in initiating shoot elongation from their axillary buds.

In promoting indigenous plant species as being suitable for small gardens or pots, propagators have to look at potential long-term problems that may arise. Upon maturity, the habit of many indigenous trees and shrubs could become too big for confined spaces

such as the urban garden. It is therefore not only important for the propagator to identify the potentially problematic species, but also to determine a means of modifying the plants' habit, so retaining its suitability as a smaller garden plant.

The dwarfing of *Combretum bracteosum* achieved promising results where an annual soil drench application of the triazole, paclobutrazol produced a compact, bushy plant with considerable visual appeal and aesthetic attractiveness. Comparing the two techniques of active dwarfing ingredient application, namely the soil drench and foliar spray methods, the former was preferred as the soil drench technique yielded results within a few weeks, whereas the foliar spray application only showed a dwarfing effect after two years. As the list of commercially available chemical compounds for enhanced flowering and vegetative growth (to mention only two) increases, the addition of one more chemical to the collection is surely not going to detract interest or potential popularity of some indigenous plant species to urban gardeners. An annual application of the paclobutrazol solution to the roots of *Combretum bracteosum*, ensures the maintenance of a compact and neat addition to any garden or pot-plant arrangement.

Therefore, the results of this research, particularly with regard to the successful propagation of *Combretum bracteosum,* coupled with the commercial appeal of its habit through dwarfing realised the stated and purported aims of this study and will hopefully promote the use of this indigenous plant species in local and overseas gardens.

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