DEVELOPMENT OF MICROPROPAGATION PROTOCOLS FOR SELECTED INDIGENOUS PLANT SPECIES

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, from January 1994 to December 1995, under the supervision of Dr. Paula Watt and Prof. Pat Berjak.

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

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ABSTRACT

The herbal medicine trade is thriving in KwaZulu/Natal with an ever-increasing number of people harvesting and trading in indigenous plants, especially those species with medicinal and/or magical properties. The number of plants harvested has increased whereas the size of the plants collected has decreased, resulting in low recruitment into wild populations. As a result of these two factors, species diversity has decreased. To this end, the aim of these investigations was to establish micropropagation protocols for the selected species i.e. *Bowiea volubilis*, *Haworthia limifolia* and *Cryptocarya latifolia*. In addition, hardening-off protocols were also developed.

The bulbous plant, *Bowiea volubilis*, was propagated via organogenesis using the inflorescence stem. Bulblet formation occurred directly without an intervening callus phase. Bulblets were produced on explants on Linsmaier and Skoog (1965) (LS) medium containing 30 $g.l^{-1}$ sucrose and either 1 $mg.l^{-1}$ BAP and 1 $mg.l^{-1}$ 2,4-D or 1 $mg.l^{-1}$ BAP and 1 $mg.l^{-1}$ NAA. Shoots and roots were induced upon transfer to the basal medium devoid of plant growth regulators. Regenerated plantlets were successfully hardened-off.

Haworthia limifolia, a succulent, was propagated via direct somatic embryogenesis using leaf material. Embryo formation was induced on a modified Murashige and Skoog (1962) (MS) medium containing 20 $g.l^{-1}$ sucrose and 1 - 5 $mg.l^{-1}$ 2,4-D. secondary embryogenesis occurred when the explants were transferred to the basal

medium supplemented with activated charcoal and devoid of growth hormones. Healthy plantlets, produced from secondary embryos, were transferred to pots and acclimatised to greenhouse conditions. A large proportion of the plantlets regenerated were vitrified and as a result, this problem was addressed by changing the medium composition or culture environment. Silica gel, when placed in the culture vessel, was the best treatment for reversal of the vitrified condition.

The establishment of leaf and nodal segment cultures of *Cryptocarya latifolia* required extensive investigation of sterilants to reduce fungal contamination. Several fungicides were tested and a successful sterilisation protocol was established. A number of media were tested for the induction of dormant axillary buds and multiplication of shoots. The best medium for both bud induction and proliferation was MS medium containing 30 $g.l^{-1}$ sucrose and 1 $mg.l^{-1}$ BAP and 0.01 $mg.l^{-1}$ NAA. Callus cultures were established on MS medium containing 30 $g.l^{-1}$ sucrose and 3 $mg.l^{-1}$ 2,4-D. These calli, however, were non-embryogenic.

Application of the established protocols and future research strategies are discussed.

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ABBREVIATIONS

NH_4^+	ammonium
BAP	benzylaminopurine
BS	Beyl and Sharma (1983) nutrient formulation
°C	degrees Celsius
Ca^{2+}	calcium (II) ions
cm	centimetre
$CoCl_2$	cobalt chloride
2,4-D	2,4-dichlorophenoxyacetic acid
GA_3	gibberellic acid
g	gram
h	hour
IAA	indoleacetic acid
K^+	potassium (I) ion
KIN	kinetin
l	litre
LS	Linsmaier and Skoog (1965) nutrient formulation
m	metre
mg	milligram
mm	millimetre
MS	Murashige and Skoog (1962) nutrient formulation
m/v	mass per volume
NAA	naphthaleneacetic acid
NaCl	sodium chloride
NaOCl	sodium hypochlorite
-ve	negative
Ni^{2+}	nickel (II) ions
pH	measure of hydrogen ion concentration
%	percent
P	probability
PGR/s	plant growth regulator/s
+ve	positive
Tween-20	polyoxyethylene sorbitan monolaurate
µg	microgram
µm	micrometer
WPM	Lloyd and McCown (1980) nutrient formulation
v/v	volume per volume
ZEA	zeatin

" Whatever befalls the earth befalls the sons of the earth. If men spit upon the ground, they spit on themselves. This we know - the earth does not belong to man, man belongs to the earth. All things are connected like the blood that unites one family. Whatever befalls the earth befalls the sons of the earth. Man did not weave the web of life; he is merely a strand in it. Whatever he does to the web, he does to himself."

CHIEF SEATTLE

" Death is one thing, an end to birth is something else."

Drs SOULE and WILCOX

Chapter 1: GENERAL INTRODUCTION

1.1. GLOBAL BIODIVERSITY

The earth is a dynamic, self-sustaining entity. Every organism on earth is linked, however tenuously, to every other. In nature, a delicate web of inter-dependence is spun among all living organisms as well as between the biosphere and geosphere. The most remarkable characteristic of living matter is its ability to self organise - in sharp contrast to the overall trend towards entropy. Information flows through the biosphere - learning and communication occur between individuals and more importantly, reproduction transfers genetic coding to new generations. However, the ever increasing human population together with the destruction of natural habitats such as tropical forests and wetlands, has resulted in a rapid decrease of natural resources. The important point though, is that it is not the lack of resources that is the issue, but how existing resources are managed.

The biological diversity of Earth is such that Man cannot survive without it. Natural resources provide the basis for life on earth. Food, water, fuels (oil and coal) and medicines are a few examples that illustrate Man's dependence on natural resources. In fact, the abuse of natural resources is self-destructive and irrational.

"Biodiversity is the total variety of life on earth. It includes all genes, species and ecosystems and the ecological processes of which they are part.." (Bibby *et al.*, 1992).

The maintenance of this biodiversity has become severely threatened. The Global Biodiversity Strategy of the International Union for the Conservation of Nature and Natural Resources (IUCN) contains many specific proposals for actions to be taken, both nationally and globally, to ensure that the world's biodiversity is preserved and is used such that resources are sustainable. The purpose of the IUCN is to complement the International Convention on Biological Diversity. This Convention requires for countries to develop national strategies or programmes for the sustainable use of biological diversity and resources.

Ultimately, the major cause of the loss of biodiversity is uncontrolled human population growth. However, the principal cause of the extinction of the world's fauna and flora, is habitat loss and degradation through large-scale clearing and burning of forests, overharvesting of plants and animals, indiscriminate use of pesticides, draining and filling of wetlands, destructive fishing practices, air pollution and the conversion of indigenous tracts of land to agricultural and urban uses. Global extinction rates have been estimated to have risen from one species per day in 1970, to one species every twelve minutes in 1992 (Wynberg, 1993). Up to 25% of the global species diversity could face extinction in the next thirty years if the present trend continues.

1.2. CONSERVATION OF BIODIVERSITY IN THE SOUTH AFRICAN CONTEXT

Very few South Africans realise that they live in a region which, in terms of plant life, is the richest in the world (Cowling *et al.*, 1989). Even when the Cape Floristic Region is excluded, southern Africa (the area including South Africa, Botswana, Lesotho, Namibia and Swaziland) still has the highest recorded plant species density in comparison with equivalent areas (Table 1.1).

Table 1.1: Number of plant species for regions of the world [adapted from Cowling *et al.*, 1989)

Region	Number of species	Area ($10^6 km^2$)	Species/area*
Southern Africa	21 000	2.57	8.1
Southern Africa (excluding Cape Floristic Region)	15 100	2.48	6.1
Brazil	40 000	8.6	4.7
India, Pakistan	20 000	4.89	4.1
Bangladesh, Burma, Australia	25 000	7.71	3.2
Europe	14 000	5.68	2.5
USA	20 000	9.36	2.1
West tropical Africa	7 300	4.50	1.6
Tropical Africa	30 000	20.00	1.5
Eastern North America	4 425	3.24	1.4
The Sudan	3 200	2.51	1.3

* Species area ratios are calculated as 10^3 plant species per 10^6 km^2.

This extreme biodiversity places a burden of responsibility on South Africans to conserve this resource. Cowling *et al.* (1989) argue that the maintenance of South Africa's plant diversity is compatible with rational and sustained use of the country's plant resources which, in turn, are important for the maintenance of economic growth.

The "preservation of biodiversity" has become a popular phrase over the last decade (Hockey *et al.*, 1994) where more and more South Africans are becoming concerned about conservation of natural resources. In addition, the actual implementation of the concept is becoming increasingly important. South Africa's biodiversity plan is such that it is appropriate with respect to the global endeavour. The South African Governments' White Paper ("Policy on a National Environmental Management System for South Africa, 1993") reaffirmed the commitment to the development of a preservation scheme. The IUCN has recommended that 10% of each country's land area should be legally protected. In South Africa, only 6% of the total land area has been formally preserved (Siegfried, 1989) by way of National Parks, Game and Nature Reserves and Conservancies. This is considerably less than several other African countries (Table 1.2).

The 1993 White Paper referred to above, proposed a policy whereby the preservation of biodiversity should be linked synergistically with sustainable development and utilisation. Thus the authors of the paper assumed that it was possible to achieve sustainable, that is economically viable, utilisation while simultaneously contributing to the preservation of biodiversity. However, Ludwig *et al.* (1993) have claimed that scientific consensus concerning exploited resources will never be attained.

Realistically, the establishment of a national biodiversity preservation strategy, although extremely important, will be difficult to manage. Furthermore, what needs to be addressed is how biodiversity in natural environments is affected by fragmented habitat structure in addition to what proportion of the diversity in natural habitats exists in man-altered environments

Table 1.2: A comparison between the percentage of protected areas within some African countries and South Africa. [Siegfried, 1989]

Country	Protected Land Area (% of total land area)
Botswana	18
Ivory Coast	15
Namibia	12
South Africa	<6
Tanzania	25

Virtually every ecosystem in South Africa has been modified or transformed by human activities leading to extensive degradation of plant resources. The exact extent of these transformations and their impact on the structure and functioning of ecosystems is difficult to determine. MacDonald (1989) loc. cit. Cowling *et al.* (1989) has estimated that 25% of South Africa has been transformed largely by cultivation (55%) and urban and peri-urban developments (26%). The effects of over-exploitation are particularly evident in forest areas where ring-barking and subsequent death of forest canopy species has resulted in the creation of gaps in the canopy such that the invasion of alien species has increased. Also evident, particularly in the Songimvelo

Game Reserve area, is the reduced species diversity in conserved areas where exploitation of medicinal plants has taken place (Mbewe, pers. comm.). Furthermore, a reduction in the carrying capacity of fruit-eating birds and economic loss due to ring-barking of trees has occurred in these areas.

Although 70% of the publicly owned terrestrial nature reserves are relatively small (Siegfried, 1989), it has been estimated that up to 74% of the plant species are preserved in nature reserves within the biomes of southern Africa (Table 1.3). However, Siegfried (1989) has pointed out that there is a distinct lack of plant inventory data for southern Africa's reserves and that there is almost no information on the extent to which reserves conserve populations of indigenous plant species.

Table 1.3: Conservation of plant species in southern African biomes (excluding Namibia and Botswana) [adapted from Siegfried, 1989]

Biome	Contribution of each biome to total sAfrican reserves (%)	Biome area reserved (%)	Occurrence of biome species in reserves (%)
Fynbos	22.9	26.3	34.8
Moist savannah	24.1	5.0	53.4
Arid savannah	17.4	12.01	49.5
Grassland	24.4	2.0	82.1
Nama-karoo	4.6	0.8	34.8
Succulent-karoo	1.9	0.8	40
Forest	4.8	88.4	no data

1.3. BIODIVERSITY AND THE MEDICINAL PLANT TRADE IN SOUTH AFRICA

1.3.1. General Background

As previously mentioned, the biodiversity within South Africa is enormous - the plant biomes (Table 1.1) alone illustrate the extent of the rich biodiversity of this country. Several plant species are endemic to South Africa. Some species are endemic to only particular areas within the country. As a result of this, several plant species have the potential of becoming endangered, for example *Warburgia salutaris*, or even extinct in the wild such as the Natal ginger plant, *Siphonochilus natalensis*. One of the reasons for the rapid loss of biodiversity is that the traditional herbal medicine trade has escalated such that many plants have become endangered or are declining - particularly in KwaZulu/Natal. Almost half a century ago, Gerstner, a priest and botanist working in the province, became concerned that the commercial sale of hundreds of species of indigenous medicinal plants was having a marked effect on the wild populations of rare species (Gerstner, 1946). He suggested that a preservation policy "merely protracted and did not prevent the lamentable process of extinction" and recommended that the solution was cultivation by nurseries that were taken up by the State and run scientifically. Since these statements were made, legislation and law enforcement have continued to dominate the approach to the problem and yet there has been a dramatic increase in the ring-barking of trees and the uprooting of other plants (Cooper, 1985; Cunningham, 1988). This has resulted in a marked decrease in the numbers of trees available for timber (for example *Ocotea bullata*). Furthermore, uprooting of plants and ring-barking of trees has resulted in an altered vegetation

structure and species diversity especially where the species involved contain active ingredients of value to both the pharmaceutical industry and the herbal medicine trade.

At present, the medicinal plant trade in South Africa is thriving (Plates 1.1 and 1.2) and major urban centres are supplied by medicinal plant gatherers from rural areas throughout KwaZulu/Natal, Eastern Cape, Swaziland and Gauteng.

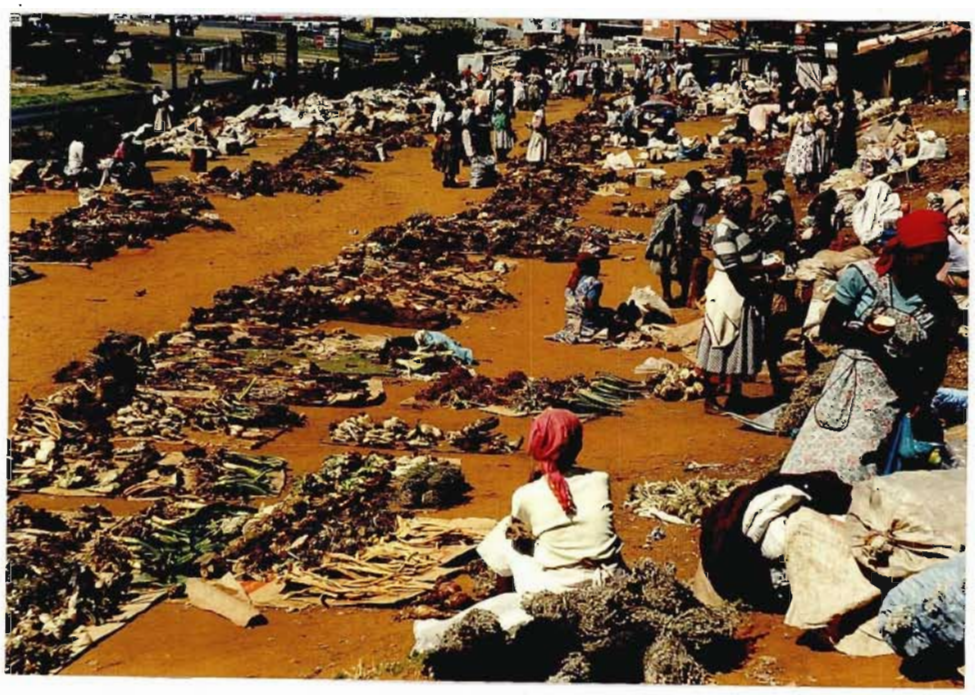


Plate 1.1: A typical medicinal plant market. (Photograph courtesy of D. von Ahleveldt)

The majority of the gatherers collect and sell medicinal plants as a means of earning a living. The increase in commercial exploitation of indigenous plants has been stimulated by three factors. Firstly, 80-85% of the rapidly expanding and urbanising black South African population consults traditional healers and uses traditional herbal remedies for both medicinal and magical purposes (Table 1.4). Secondly, traditional healers are more numerous and more accessible than western practitioners especially in rural areas. Thirdly, high rates of unemployment and little formal education are common in KwaZulu/Natal, and are linked to even greater usage of indigenous plants (Cunningham, 1988).

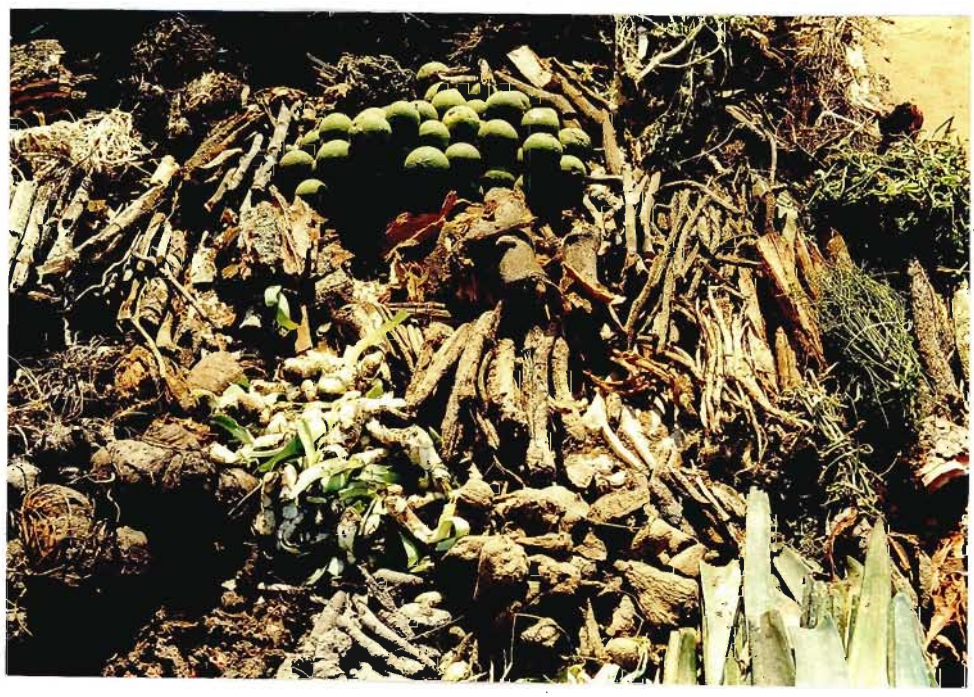


Plate 1.2: A selection of plant parts used in traditional medicines. (Photograph courtesy of D. von Ahleveldt)

1.3.2. Supply

Over four hundred indigenous and twenty exotic species are commercially sold as herbal medicines (Cunningham, 1988). The species vary widely in their geographical distribution, growth rate, growth form and population biology. The demand for species which have high growth rates, set seed easily and are widely distributed is easily met (for example *Artemisia afra* and *Catunaregam spinosa*). However, species with a low population density, slow growth rate and sensitivity to methods of harvesting (for example ring-barking) are extremely vulnerable to over-exploitation. In addition to these factors, there has been rapid decline in areas of natural indigenous vegetation. In KwaZulu/Natal, the primary reasons for this has been the spread of urban development and increased afforrestation and agriculture (Cunningham, 1988).

1.3.3. Demand

The demand for herbal medicines has increased with cultural and socio-economic change and population growth (Cunningham, 1988). In the mid to late 1980s, the total black population in KwaZulu/Natal, was estimated at over 5 million people (Central Statistics Service, 1987). The projected annual increase was 2.41% in 1985 (Grobbelaar, 1985) and in the Greater Durban area, has risen by 9% per annum (Anon, 1986). A large proportion of the population consult traditional healers as mentioned above. As a result of the steady increase in population, the demand for traditional medicines has increased and therefore an unprecedented growth has occurred in commercial harvesting and the sale of traditional medicines. According to Cunningham (1988), there were only two herb traders in Durban in 1929. In sixty years, this number had risen to over a hundred registered traders belonging to the

Natal Herb and Traditional African Medicines Traders Association (Cunningham, 1988). Informal herb traders number approximately five hundred in the major medicinal plant markets in Durban (Victoria Street and Isipingo Markets) (Sibaya, pers.comm.).

1.3.4. Commercial Harvesting

Large quantities of the most important medicinal plant species are sold each year by herb traders in KwaZulu/Natal (Table 1.5) which is probably causally related to a sharp decline in the plant numbers of important species (for example *Alpeidia amatymbica, Erythrophleum lasianthum, Mondia whitei* and *Warburgia salutaris*) in wild populations i.e. a loss of biodiversity. This has led to the production of a "red list" of species which are described as declining, vulnerable or endangered (Table 1.6) due to over-exploitation A comparison of the species presented in Tables 1.4, 1.5 and 1.6 reveals that the majority of plants most commonly traded for use as herbal medicines are on the "red list".

1.3.5. Legislation

Registration of herbalists, based on the recommendation of hereditary chiefs in whose area the herbalist resided was accomplished in Natal under the Native Code Law of 1891 and in Zululand under Section 194 of the Zululand proclamation No. 7 of 1985.). In KwaZulu/Natal, herb traders require a General Dealers Licence and Patent Medicines Licence to sell unprotected flora (Cunningham, 1988). Additionally, permits are required to sell protected species (Williams, 1992). Gatherers are permitted to collect plants only if permits are obtained from the Natal Parks Board,

and the provinces of South Africa have legislation concerning the indiscriminate removal and damage of indigenous vegetation (Williams, 1992). In KwaZulu/Natal, for example, protected indigenous plants many of which are important medicinal plants, may not be imported into or exported out of the province. However, it is known that indigenous plants are gathered from outside the province and illegally transported to markets (Victoria Street and Isipingo Markets in the Greater Durban area) where they are sold.

Present legislation in KwaZulu/Natal aims to prevent: (1) Exploitation of indigenous plant species in State forests (Forest Act No. 122, 1984, KwaZulu Forest Act No. 15, 1980, KwaZulu Nature Conservation Act No. 8, 1975 and the Natal Provincial Ordinance of 1974)

(2) the exploitation and sale of specially protected species (Natal Provincial Ordinance of 1974)

(3) hawking, including hawking of medicinal plants (Natal Ordinance 11 of 1973)

(4) the sale of poisonous substances by herb traders (Medicines and Related Substances Control Act No. 101 of 1965) and

(5) the practising of unlicensed herbalists or any practising diviners (Suppression of Witchcraft Act, 3 of 1957)

According to Cunningham (1988) the extensive legislation existing in KwaZulu/Natal has failed to achieve these objectives.

Table 1.4: The uses of traditional medicine types sold in the Victoria Street and Isipingo Markets in the Greater Durban area [Adapted from Ntuli (1994), Honours project]

Botanical name	Zulu name	Use
Acokanthera oppositifolia	Khwangu,u-	Asthma, cough
Albizia adianthifolia	Gadankawu,um-	Lice
Andrachne ovalis	Membeso,u-	Pubic lice
Berchenia discolor	Madlozana,u-	Good luck
Boophane disticha	Ncotho,i-	Blood purifier
Bowiea volubilis	Gibisila,i-	Blood purifier
Calamus spp.	Kalamuzi,u-	Asthma, cough
Cinnamomum camphora	Loselina,u-	Charm women
Discorea sylvatica	Fudu,u-	Pain-killer
Ekebergia capensis	Nyamathi,um-	Good luck
Eriostema cordatum	Bangalala,u-	Sexual stimulant
General term[*]	Lawu,ubu-	Good luck
General term[*]	Mbiza,i-	Blood purifier
General term[*]	Ntelezi,i-	Ward off evil spirits
Harpephyllum caffrum	Gwenya,um-	Pimples
Helichrysum spp.	Mpepho,i-	Burns, speak to ancestors
Knowltonia bracteata	Vuthuza,um-	Pubic lice
Microgramma lycopodioides	Khambi lezintwala,i-	Pubic lice
Ochna spp.	Madlozana,u-	Good luck
Ocotea bullata	Nukani,u-	Sorcery
Osteospermum imbricatum	Mashiqolo,u-	Tonic
Pentanisia prunelloides	Cishamlilo,i-	Swollen feet
Pittosporum viridiflorum	Fusamvu,um-	Bile tonic
Podocarpus henkelii	Ngqonqqozi,aba-	Sorcery
Protorhus longifolia	Nhluthe,i-	Sex stimulant
Schlechterina mitrostammatoides	Nhlalanyosi,i-	Good luck
Scilla natalensis	Nguduza,i	Purgative
Sclerocarya birrea	Ganu,um-	Pimples
Secamore gerradi	Gobandlovu,u-	Stroke, epilepsy
Senecio spp.	Nsukumbili,u-	Sores
Urginea altissima	Mahlanganise,u-	Fractures

Table 1.5: Quantities of medicinal plant species sold (in 50 kg bags/year) by approximately fifty herb traders in the KwaZulu/Natal region. The total annual sales for the entire region can be approximated as 400% greater than the amounts listed. The parts used are abbreviated as follows: bb = bulbs, r = roots/rhizomes, l/st leaves and stems, bk = bark, wpl = whole plant, fr = fruits. (Adapted from Cunningham, 1988).

Species	Part Used	Demand (Number of bags)
Scilla natalensis	bb	774
Eucomis autumnalis	bb	581
Aleipidea amatymbica	r	519
Adenia gummifera	st	459
Albizia adianthifolia	bk	424
Clivia miniata	bb	397
Clivia nobilis	bb	
Pentanisia prunelloides	r	343
Senecio gregatus	l/st	340
Senecio serratuloides	l/st	340
Gunnera perpensa	r	340
Rapanea melanophloeos	bk	327
Dioscorea sylvatica	wpl	326
Warburgia salutaris	bk	315
Bersama stayneri	bk	295
Bersama tysoniana	bk	295
Kalanchoe crenata	l/st	284
Bowiea volubilis	bb	257
Trichilia dregeana	bk	252
Trichilia emetica	bk	
Turbuia oblongata	r	249
Rhoicissus tridentata	r	244
Bulbine latifolia	bb	240
Ocotea bullata	bk	234
Stangeria eriopus	r	233
Cryptocarya myrtifolia	bk	228
Cryptocarya latifolia	bk	
Anemone fanninii	r	227
Eucomis biclor	bb	224
Rhus chirindensis	bk	222
Helinus integrifolius	st	222
Schotia brachypetala	bk	220
Vernonia neocorymbosa	l/st	216
Dioscorea dregeana	wpl	212
Ornithogallum longibractealum	bb	208
Erythrophleum lasianthum	bk	201
Solanum aculeastrum	fr	198
Curtisia dentata	bk	197
Microgramma lycopodioides	wpl	190
Kalanchoe sp.	l/st	190
Aspilia natalensis	wpl	186
Dietes iridioides	r	186
Callilepis laureola	r	181
Helichrysum actuatum	r	179

Table 1.6: Indigenous plant species that are considered to be a high conservation priority in the KwaZulu/Natal region.
Declining: Species that were recently widespread but are likely to become vulnerable and to continue to decline if destruction of wild populations continues
Vulnerable and Declining: Species that were more widespread in the past but are now likely to become endangered if destruction of wild populations continues
Endangered: Species considered to be in danger of extinction if destruction of wild populations continues
Extinct: No wild populations in former localities although the species may exist under cultivation.
(Adapted from Cunningham, 1990)

Status	Species
Declining	*Mondia whitei*
	Schlechterina mitostemmatoides
	Acridocarpus natalitus
	Andrachne ovalis
	Garania gerrardii
	Cryptocarya latifolia
	Erythrophleum lasianthum
	Prunus africana
	Cassine transvaalensis
	Balanites maughamii
	Harpephyllum caffrum
	Aleipidea amatymbica
	Anemone fanninii
	Cassipourea gerradii
	Cassipourea flanaganii
	Pterocelastrus echinatus
	Pterocelastrus rostratus
	Pterocelastrus tricuspidatus
	Cassine papillosa
	Bowiea volubilis
	Bersama swynii
	Scilla natalensis
	Eucomis autumnalis
	Eucomis bicolor
	Eulophia cucullata
	Stangeria eriopus
Vulnerable and Declining	*Dioscorea sylvatica*
	Bersama tysoniana
	Ocotea bullata
	Ocotea kenyensis
	Curtisia dentata
	Pleurostylia capensis
	Faurea macnaughtonii
	Haworthia limifolia
	Mystacidium mellarii
Endangered	*Warburgia salutaris*
	Siphonochilus aethiopicus
Extinct (in the wild)	*Siphonochilus natalensis*

1.4. TISSUE CULTURE AND ITS APPLICATIONS TO THE CONCEPT OF SUSTAINABLE RESOURCE UTILISATION

The loss of biodiversity is of major environmental concern at the present time, whether this relates to the destruction of the tropical rain forests, for example, or the threat which faces natural ecosystems as climates change due to global warming (Jackson and Ford-Lloyd, 1990). It is important that, in order to survive and continue to evolve, plant and animal species maintain genetic diversity.

In the Greater Durban area, traditional medicine is used by about two thirds of the rapidly growing population (Nichols, 1990) and the management of natural areas within the city limits has emphasised the severe collection/harvesting pressure on many species of medicinal plants, many of which are on the verge of extinction. As mentioned above, over-exploitation of medicinal plants has arisen through several factors, but it is important to realise that this exploitation is further enhanced because many species have low seedling recruitment. Many of the species used in traditional medicines are either slow growing, therefore reaching maturity (and thus sexual propagation) at a much later stage than other species. Furthermore, the numbers of seeds set may be low, recalcitrant, seeds may have a very short life span, or the seeds which are produced may not be viable.

Alternative techniques for artificially propagating such species are a means toward relieving pressure on wild plant populations. One such technique, with enormous potential is micropropagation. This method provides a way of mass-producing a

particular species using any part of the plant (for example leaves, stems and/or inflorescence) as the starting material since all plant cells are totipotent, i.e. a single cell has the potential to develop into a complete new plant. This has obvious implications for the mass propagation of exploited species and hence for relief of harvesting pressures on wild populations of these species.

1.5. ASPECTS OF *IN VITRO* METHODS REQUIRED FOR THE ESTABLISHMENT OF MICROPROPAGATION PROTOCOLS FOR INDIGENOUS SPECIES

1.5.1. Aspects of cell and tissue culture

Individual cells have the ability to grow and divide independently due to the phenomenon of totipotency whereby a cell, under specific conditions, is able to regenerate completely thus forming an entire organism (Mantell *et al.*, 1985; Duncan and Widholm, 1986; Lindsey and Jones, 1990; Allan, 1991). In this manner, a large number of clones may be produced from a small piece of parent material (somatic cells or tissue) given the appropriate conditions. Organised development in both plants and animals is complex and is influenced by both internal and environmental factors (Thorpe, 1983; Warren, 1991).

There are two routes whereby plants may be regenerated: 1) somatic embryogenesis, by which fully formed embryos are generated and consequently induced to germinate or 2) organogenesis, the production of shoots followed by root formation (Evans *et al.*, 1981; Thorpe, 1983; Ammirato, 1986). The two routes may occur directly from the explant or indirectly, via a callus stage. Callus is a mass of undifferentiated cells formed by transferring a sterile explant onto a nutrient medium supplemented with plant growth regulators (Constable, 1984; Duncan and Widholm, 1986; Collin and Dix, 1990; Allan, 1991). The cells have a low level of organisation and can theoretically be maintained indefinitely by routine subculture onto fresh nutrient medium (Collin and Dix, 1990; Allan, 1991). Manipulation of the medium may allow

whole plants to be regenerated from cultured cells by either direct or indirect routes of differentiation.

The formal definition of somatic embryogenesis is the non-sexual development of a structure in which shoot and root poles develop in the same temporal sequence as that seen in a zygotic embryo (Haccius, 1978; Tulecke, 1987, Schwedimann *et al.*, 1988, 1990; Warren, 1991). An alternative description is that of Williams and Maheswaran (1986) and Wann (1988) who have defined somatic embryogenesis as the process by which haploid or diploid somatic cells develop into individual plants through characteristic embryological stages without the fusion of gametes. Of the advantageous applications of somatic embryogenesis, perhaps that which is most significant is the potential for mass regeneration (Sharp *et al.*, 1982; Lutz *et al.*, 1985; Ammirato and Styer, 1985) due to the ability to produce unattached units rapidly and at high density (Harrell *et al.*, 1992). In addition, there is also the possibility of long-term storage (Kitto and Janick, 1985; Lutz *et al.*, 1985; Ammirato, 1986). The presence of both root and shoot primordia in the same unit, the possible achievement of rejuvenation, and the potential for dormancy induction are additional features which make somatic embryogenesis a particularly useful method for *in vitro* propagation. Furthermore, because of the large numbers of somatic embryos that can be derived from a small piece of explant tissue, this method is also useful when conservation of a genotype is important as is in the present investigations.

Another culture system that has commonly been used for the *in vitro* propagation of plant tissues, is direct organogenesis via axillary bud proliferation (Table 1.6). The steps involved are shoot multiplication, elongation and rooting of generated shoots (Evans *et al.*, 1981; Flick *et al.*, 1983; Thorpe, 1983; Ammirato, 1986) and as a result of the high yields obtained and the production of clones (Nashar, 1989). This method is used in large-scale micropropagation of many ornamental (Yeoman, 1986), agronomic (Hu and Wang, 1983) and forest species (Biondi and Thorpe, 1981; Durzan, 1984).

Other culture systems used in the regeneration of plants include the production and culture of protoplasts (Vasil *et al.*, 1990; Scarpa *et al.*, 1993), anthers (Hu and Zeng, 1984; Pugliesi *et al.*, 1993; Thenugane *et al.*, 1994), pollen grains (Yeoman, 1986), ovaries (Agrawal and Gebhardt, 1994) and inflorescences (Balan, 1994; Kalia and Crisp, 1994).

1.5.2. Factors affecting *in vitro* culture systems

There are several factors which affect culture systems. Each does not generally exert an effect on its own, however, and several factors may act synergistically to produce the desired effect. Both physical elements and chemical constituents are important to the success *of in vitro* culture systems.

1.5.2.1. Choice and preparation of explants

The major requirements of an effective explant tissue is a propensity for cell division and morphogenetic plasticity (Warren, 1991). These criteria are usually met by

meristematic or rapidly growing tissues whereas mature tissues are morphogenetically determined and are not as susceptible to dedifferentiation (Durzan, 1984; Warren, 1991). Callus is able to develop from any explant although the explant type is dependent on the type of research to be undertaken (Allan, 1991). Plant parts that have been used as explants include nodal segments (Cortezzi Graça and Mendes, 1989; Blomstedt *et al.*, 1991; Bhat *et al.*, 1992), root sections (Sood, 1994), leaf pieces (Bolyard *et al.*, 1991; Jäger *et al.*, 1993), inflorescences (Chen *et al.*, 1985; Vasil *et al.*, 1990; Kalia and Crisp, 1994), pollen (Dodds and Roberts, 1985; Yeoman, 1986), cotyledons (Jang and Tainter, 1991; Griga, 1993; Pugliesi *et al.*, 1993), hypocotyls (Scarpa *et al.*, 1993; Lee *et al.*, 1994) and zygotic embryos (Cao *et al.*, 1992). Factors such as source of the tissue, age, season in which the tissue was obtained, explant size and quality affect reproducibility of results and therefore require analysis. Selected explants must be surface sterilised and placed on the appropriate medium (Thorpe, 1980; Allan, 1991). In addition, explant orientation and contact with the nutrient medium may also need to be taken into consideration (McClelland and Smith, 1990; Allan, 1991; Warren, 1991).

1.5.2.2. Maintenance of aseptic cultures

Surface sterilisation procedures using common household disinfectants may be sufficient in eliminating surface contaminants. However, contamination may still occur in cultures as a result of endogenous bacterial and fungal propagules within plant tissues (Gordon and Brown, 1988; Warren, 1991). Antimicrobial agents such as antibiotics (Kneifel and Leonhardt, 1992) or fungicides (Shields *et al.*, 1982) may be used if they are broad-spectrum (or specific to a contaminant) and are non-toxic to the

plant material. Furthermore, maintenance of aseptic cultures also requires that all operations are carried out under sterile conditions using sterile equipment and nutrient media (Dodds and Roberts, 1985).

1.5.2.3. The culture environment

Diminished plant quality and low survival rates *ex vitro* may be observed (Ziv, 1991; DeBergh *et al.*, 1992). Various factors involved in plant growth and an understanding of these factors may facilitate the improvement of plant quality and prevention of abnormalities that may arise as a result of *in vitro* culturing. Consequently, many factors require consideration when *in vitro* studies are undertaken. Furthermore, these factors are all interlinked, resulting in an extremely complex situation.

Chemical factors

Numerous media formulations for the culture of many plant species have been formulated and published (George *et al.*, 1988). Plant culture media are based on different concentrations of essential organic and inorganic salts, a carbon source and growth hormones. The effects of different concentrations of carbohydrates and nitrogen, essential in culture systems, have been reviewed by Thompson and Thorpe (1987) and Kirby *et al.* (1987), respectively. Carbon is an essential component since most culture systems are heterotrophic. It is normally provided in the culture medium as sucrose, although the media may sometimes be supplemented with the monosaccharides (Atanassov and Brown, 1984; David *et al.*, 1984; Sundberg and Glimelius, 1986). Many species grow on a wide range of media: however, some

require specific additives such as coconut milk or casein hydrolysate (Thorpe, 1980; Ammirato, 1986; Allan, 1991).

Plant growth regulators are important in induction and control of morphogenesis (Ammirato, 1986). A specific plant growth regulator may have a wide range of physiological effects in different plants and apparently in different parts of plants, depending on the presence or absence of endogenous hormones within the plant tissue (Minocha, 1987). In addition, genetic make-up and physiological status of the tissue may be factors involved. It is important to note that plant growth regulators do not elicit a response alone: it is rather a response of the plant tissue to a particular balance or ratio between different growth regulators that is important (Ammirato, 1986). The type of regeneration that is attempted determines the optimum growth regulator regime to be utilised (Warren, 1991). The importance of such growth regulators as abscisic acid (ABA), benzylaminopurine (BAP), indoleacetic acid (IAA), kinetin (KIN), naphthylacetic acid (NAA) and zeatin (ZEA) in induction and morphogenesis has been reviewed extensively (Thorpe, 1983; Ammirato, 1986; Minocha, 1987; Reynolds, 1987). The balance between auxins (IAA, NAA) and cytokinins (BAP, KIN, ZEA) determines whether disorganised growth occurs or whether shoots and roots develop (Ammirato, 1986). Generally, high auxin concentrations promote the development of disorganised growth such as callus while the ratio between auxin and cytokinin influences the balance between shoot and root growth. High cytokinin levels have been found to inhibit root formation (Warren, 1991).

A further problem often encountered in the initial culture stages is the browning and eventual death of the explant tissue due to the production of polyphenolics. These organic compounds arise as a result of wounding (Tulecke, 1987). The problem is often overcome by the addition of adsorbents such as activated charcoal, polyvinylpyrrolidone (PVP), or antioxidants such as ascorbic acid, to the nutrient medium (Tulecke, 1987; Warren, 1991).

Physical factors

Chemical constituents in the culture environment are extremely important and culture systems may be beneficially or adversely affected by them. However, the correct physical environment is also vital since the various parameters may have a profound effect on the success of the culture system in question (Hughes, 1981; Warren, 1991).

An important environmental factor which needs to be considered is light. It has a strong effect on plant morphogenesis in *in vitro* culture and not only is the absence or presence of light critical, but also the physical characteristics such as wavelength, intensity, quality and photoperiod (Thorpe, 1980; Ammirato, 1986; Kozai, 1991). *In vitro* cultures have different radiant energy requirements compared with autotrophic plants since the former do not photosynthesise (Thorpe, 1980). However, often the light required for photomorphogenic events (Thorpe, 1980) of tissue cultured material is not considered or the placement of light sources is incorrect. For example, lateral illumination on two sides of the culture vessel has been found to promote plant growth and produce better plant shape due to the uniformity of light on the culture system and thus improved interception of light by the leaves (Kozai, 1991).

Most plants respond to temperature changes and some plants require different temperature regimes within the *in situ* environment. It can therefore be assumed that *in vitro* culture systems also require particular temperature regimes for optimum growth. Generally, cultures are maintained at 25 °C (Thorpe, 1980). Kozai (1991) has shown that even in a controlled temperature room, there is a temperature fluctuation of 1 °C between the light and dark periods in culture, but that the dry and fresh weights of plants are not affected by this. Obviously, a range of suitable temperatures and the temperature optimum will vary from species to species (Hughes, 1981; Ammirato, 1986). Temperate species will have a lower temperature optimum than tropical species, for example, and this must be taken into account in experimental design.

The way in which cultures are grown may affect morphogenesis markedly (McClelland and Smith, 1990). Factors affecting morphogenesis may include: culture vessel (McClelland and Smith, 1990; Kozai, 1991), relative humidity within the vessel (DeBergh *et al.*, 1992), gaseous exchange between the culture vessel and the external environment (Kozai, 1991) and the nature of the gelling agent used (Gorinova *et al.*, 1993).

The type of culture vessel and vessel closure affects the gaseous composition and lighting of the micro-environment, and hence *in vitro* plant growth and vitrification (McClelland and Smith, 1990; Kozai, 1991). In order to prevent contamination and therefore loss of material by micro-organism proliferation, closed containers are used in tissue culture. As a result, the size of the headspace and the gaseous composition

(relative humidity and carbon dioxide levels) in the headspace of the culture vessel is markedly different from ambient conditions. The high relative humidity gives rise to the phenomenon of vitrification (Paques *et al.*, 1985; DeBergh *et al.*, 1992) frequently observed in woody and some herbaceous plants. Vitrification occurs as a result of changes in metabolic and physiological function and is often associated with poor explant material and incorrect culture environment (for example high humidity or poor gaseous exchange). The poor growth of vitrified shoots is accompanied by low rates of multiplication, rooting and survival after transfer to soil whereupon plantlets become extremely susceptible to dehydration and infection (Gaspar *et al.*, 1987). The occurrence of vitrification is random and practical strategies for its avoidance are important.

1.5.3. Acclimatisation of regenerated plants

The clonal propagation of plant species can be achieved through explant establishment, shoot multiplication and development, and finally acclimatisation or hardening off, followed by establishment of plants in the field (Murashige, 1974). The ultimate success of micropropagation depends on the survival of plantlets and good performance under natural conditions (Bhojwani and Dhawan, 1989). Successful acclimatisation is influenced by the conditions during the propagation, rooting and acclimatisation stages (Van Telgen *et al.*, 1992). Hardening-off involves the exposure of plants to reduced relative humidity (Ziv, 1986) for a period of time until the plantlets are able to withstand the conditions of the ambient environment. *In vitro* culture conditions which promote rapid growth and shoot proliferation often result in physiologically abnormal plants which do not survive *ex vitro* (Van Telgen *et al.*,

1992). Often, abnormal leaf physiology, malfunctioning stomata and a reduction in cuticular waxes (Ziv, 1986) are observed, resulting in desiccation of plantlets upon transfer from culture. Thus, the hardening-off procedure is extremely important for *ex vitro* plantlet survival.

1.6. THE APPLICATION OF TISSUE CULTURE TECHNOLOGY TO *EX SITU* PLANT GENETIC CONSERVATION

1.6.1. *In situ* and *ex situ* conservation

In situ conservation is used for threatened species in their natural habitats, for example, in national parks or nature reserves (Krogstrup *et al.*, 1992). An advantage of this is the continuation of natural selection so that species evolve with respect to the natural physiological and biological environment. *Ex situ* conservation of plant species occurs outside the natural habitat (for example, in botanical gardens). The major disadvantages are that natural evolution is impeded and artificial selection pressures are imposed on the species. However, where the natural environment has been destroyed and the species is endangered in the natural situation (as discussed in 1.2), *ex situ* conservation provides the only possibility of genetic conservation.

Tissue culture technology presents a complement to the methods usually applied in *ex situ* conservation and further, broadens the scope of *ex situ* conservation. Figure 1.1 is a schematic representation of the use of tissue culture in *ex situ* conservation.

In vitro methods are a valuable tool in conservation strategies. This is particularly true for species with recalcitrant seeds (seeds which cannot be stored at low moisture contents [Roberts and King, 1980]) or tree species which require a large amount of space if they are to be field grown as conservation stands where genetically important material is maintained in the field. In this case, the use of *in vitro* storage in terms of limited growth cultures or cryopreserved embryonic axes would ensure cost-effective *ex situ* conservation. Furthermore, when germplasm can be retrieved from storage and multiplied rapidly by micropropagation methods, it is available throughout the year.

The disadvantages of this approach are that there are no universally applicable methods for storage or micropropagation and different protocols need to be developed for each species which is labour-intensive and expensive.

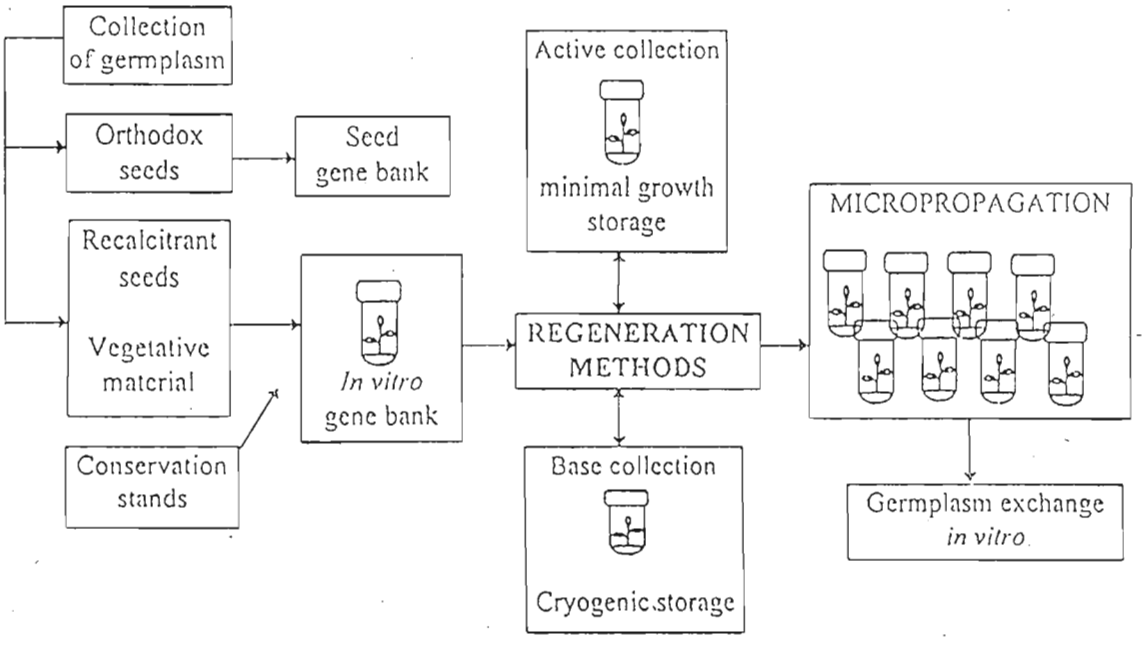


Figure 1.1 : The application of tissue culture in *ex situ* conservation [adapted from Krogstrup *et al.*, 1992]

1.6.2. Methods of *ex situ* conservation.

Ex situ germplasm acquisitions are maintained as either active or loose collections (Hawkes, 1987). An active collection requires methods of storage that retain viability for short periods and is normally maintained as a field or greenhouse collection or as a conservation stand. Regular regeneration and multiplication maintain some genetic diversity even though the collections are exposed to artificial selection. The maintenance of active collections as living plants in the field or greenhouse is extremely labour-intensive and expensive. In addition, there may be substantial loss of material due to pests or diseases. In contrast, base collections are intended for long-

term conservation of germplasm. Maintenance of base collections requires methods that retain viability for long periods.

1.6.3. Seed storage

Seed storage is the most common means of storing plant germplasm. Low temperature is one of the main factors that determines seed viability in storage but is not applicable to all species. Differing physiological responses to desiccation and low temperature have resulted in the division of seeds into two groups i.e. "orthodox" and "recalcitrant" seeds (Roberts, 1973), while another category, that of intermediate seeds, has been more recently defined (Ellis, 1991). Seeds which may be dried to low moisture contents without losing viability and which can tolerate freezing temperatures, are termed orthodox. Recalcitrant seeds are those seeds which lose viability at higher moisture contents and cannot be stored at low temperatures (Roberts and Ellis, 1977; Berjak, Farrant and Pammenter, 1989). The seeds of many tropical recalcitrant species do not tolerate relatively cool temperatures, well above freezing (for example, 10 °C is limiting for seeds of cocoa, rambutan and camphor). Other cases where seed storage is not a viable option, include species that do not produce many, or viable seeds regularly. Included here are plants with periodic seed production, for example, many forest tree species where seed set is low or where seed produced is not viable. Thus, for a number of plants and situations, seed storage is not a feasible option. *In vitro* or tissue culture methods are therefore an important alternative, particularly where a species is economically important or faces the threat of extinction.

1.6.4. *In vitro* storage methods

There are three approaches to *in vitro* storage: 1) storage of actively growing cultures, 2) minimal growth storage and 3) cryopreservation (Krogstrup *et al.*, 1992). The first method requires that plant material is maintained as actively growing cultures. This requires monthly subculturing with a possible risk of loss of material through contamination or decay. The advantage of this method, however, is that plantlets may be rapidly multiplied by micropropagation. Minimal growth storage exposes tissue cultures to growth limiting chemical and/or physical factors such as growth retardants, reduced temperatures and reduced pO_2 (Baucher *et al.*, 1989, Lizaragga *et al.*, 1989, Engelmann, 1990). Cryogenic storage is based on the reduction and subsequent cessation of metabolic functions in biological material while retaining viability by applying ultra-low temperatures (-80°C - 196°C) (Kartha, 1985). When plant cells are exposed to ultra-low temperatures, cellular metabolic activities cease, as do most deleterious reactions, due to the unavailability of water and consequently resultant genetic aberrations are minimised. Cell suspensions (Withers, 1985), shoot and meristem cultures (Kartha, 1985) and somatic embryos (Withers, 1980) have been shown to have the potential for cryostorage.

1.7. AIMS OF THE INVESTIGATIONS

The limiting factors of *in vitro* storage is the development of *in vitro* plant regeneration protocols for different species. However, once these have been established for the plant species in question, a large number of the species concerned can be generated, thus ensuring that the existing population numbers are increased. This investigation is aimed to address problems of *in vitro* propagation of three vulnerable and declining (Table 1.5) plant species: *Bowiea volubilis*, *Haworthia limifolia* and *Cryptocarya latifolia*. The plants are all heavily utilised in the herbal medicine trade in KwaZulu/Natal and are under threat from over-exploitation by the trade. The primary objective was to establish aseptic cultures from field grown material and to develop micropropagation protocols (via embryogenesis and/or organogenesis) for each species. As the success of any tissue culture system requires that regenerated plantlets are acclimatised to ambient conditions so that the plants may be reintroduced into wild populations, the second objective was to establish hardening-off protocols for successfully regenerated plantlets of the species under study.

Chapter 2: ESTABLISHMENT OF MICROPROPAGATION PROTOCOLS USING THE INFLORESCENCE OF *Bowiea volubilis*

2.1. INTRODUCTION

2.1.1. Description of *Bowiea volubilis*

Bowiea species belong to the Liliaceae. There are two species of *Bowiea, B. volubilis* and *B. garipiensis,* which are endemic to South Africa, *B. garipiensis* having been more recently described by van Jaarsveld (1983). *Bowiea volubilis* (Plate 2.1) is a geophyte occurring in the central and eastern regions of South Africa, and is described in detail by Phillips (1952) and Dyer (1964). According to those authors, the roots are fleshy and white and up to 5 mm in diameter, while the bulb is globose and up to 150 mm in diameter. The bulb may be subterranean or exposed, the exposed parts being green. The leaves are lanceolate, canaliculate up to 350 mm long and 5 mm in diameter, and wither slightly prior to flowering. The stem producing the annual inflorescence can be up to 3 m long and appears during the summer months. The inflorescence is pendulous and scrambling, highly branched (almost dichotomously so) and is basally flexuose. Branchlets are up to 500 mm long and 2 - 3 mm in diameter. The flowers are inconspicuously green, 25 mm in diameter with erect stamens, 5 mm long and a superior ovary which is broadly conical, and 5 mm at the base.

Plate 2.1: The growth habit of *Bowiea volubilis*. Note the extensive branching of the inflorescence stem. (bar: = 62.5 mm)

2.1.2. Previous studies on *Bowiea volubilis*

Bowiea volubilis has been studied since the late nineteenth century and a number of reports on the biology of this species have appeared in the literature. Table 2.1 gives an indication as to the diversity of fields in which *B. volubilis* has been studied.

Table 2.1: Some reported investigations into the biology of *B. volubilis*

Author/s	Field of Study
Hooker (1867) loc. cit. Bullock (1978)	Ecology
Irmisch (1979)	Chemistry
Karzel (1924)	Vegetative regeneration
Curson (1928)	Chemistry
Bucur (1929)	Anatomy and Physiology
Dyer (1941)	Taxonomy
D'Amato (1949)	Cytology
Stenar (1949)	Anatomy and Physiology
Jha and Sen (1986)	Micropropagation
Cooke *et al.* (1988)	Micropropagation

2.1.3. The uses of *Bowiea volubilis* in South Africa

The bulb of *B. volubilis* has long been known to be poisonous (Watt and Breyer-Brandwijk, 1962). Nevertheless, the bulb, usually mixed with other constituents, forms an important part of traditional medicine in South Africa. Preparations are purported to have both medicinal and magical properties. Some of the methods of preparation and presumed modes of action of these remedies are described in Table 2.2.

Table 2.2: Methods of preparation of *Bowiea volubilis* and the medicinal or magical attributes to which they have been ascribed in South Africa.

Preparation	Mode of Action	Reference
Roasted, powdered and added to water	Mild purgative in small doses. Taken in excess results in poisoning.	Watt and Breyer-Brandwijk (1962)
Ground bulb	Remedy for dropsy Treatment against female infertility Aphrodisiac	Watt and Breyer-Brandwijk (1962)
Extracted juices	Skin lotion for treating the sick	Mkhize, pers. comm.
Decoction of the bulb	Lotion for painful eyes Sprinkled on "impis" causes the enemy to flee	Watt and Breyer-Brandwijk (1962)
Outer scales of bulb	Remedy for ascites	Watt and Breyer-Brandwijk (1962)

Several cases of poisoning in Kwazulu/Natal have resulted in court cases (Watt and Breyer-Brandwijk, 1962). The poisoning has often been attributed to excess dosages of bulb preparations. According to those authors, symptoms of overdosage are vomiting and purging, cramps and eventually death, 24 - 48 hours after ingestion. *Post mortems* have indicated the congestion of organs, irritation of the gastro-intestinal tract and heart-stoppage. Watt and Breyer-Brandwijk (1962) have reviewed the research of several workers who have isolated compounds which may be implicated in reactions with the body organs. The above ground parts of the plant have also been reported to be toxic although they do not appear to exhibit a cardiac poison action (Curson, 1928).

2.1.4. Modes of propagation

Over the last decade, there has been considerable interest in the development of tissue culture techniques for the rapid propagation of healthy stocks of bulbs and new varieties of liliaceous plants (Hussey, 1980). Traditional methods of vegetative propagation of bulbs such as daughter-bulb formation, scooping, twin-scaling and chipping can and have been employed in nurseries. However, it may require decades before commercial quantities of a particular bulb species from a single plant become available. In that time, material considered virus-free may become reinfected or new varieties may be released from breeding programmes. In addition, many bulbs (for example *Tulipa* spp.), cannot be propagated vegetatively other than by the natural production of daughter bulbs. This process provides an extremely slow rate of multiplication. Alderson and Rice (1986) have reported that only two thousand bulbs can be propagated over a period of ten years from a single bulb. Thus, there has been a distinct need to investigate the potential of *in vitro* propagation systems for such plants. Extensive studies have been done on the micropropagation of bulbous species and several established protocols exist. Some of the reported studies are listed in Table 2.3. Explant source and medium formulations vary markedly among species, such that micropropagation of a particular species requires specific conditions for successful regeneration (Table 2.4). Most of the species are economically important in industry (for example *Allium sativum* and *Amaranthus hypochondriacus*) or where wild populations of the species are under threat from over-exploitation.

Very little is known about the natural propagation of *B. volubilis*. However, it has been reported that few seeds are set (Dyer, 1964) and hence progeny numbers are low.

Bowiea volubilis has been previously propagated using tissue culture techniques (Jha and Sen, 1986, Cooke *et al.*, 1988). Jha and Sen (1986) obtained plantlets using suspension cultures with explant material obtained from bulb scales. However, micropropagation from suspension cultures is expensive and labour intensive. Cooke *et al.* (1988) successfully produced *B. volubilis* in culture using bulb scale material as explants. Using the bulb is highly destructive since the parent plant is destroyed, thus, this is not an ideal option in terms of conservation of a rare and endangered species which is heavily exploited (Section 1.3.5).

2.1.5. Aims of the investigation

As discussed previously (Section 1.5), micropropagation techniques provide a means of regenerating plants and ultimately a strategy for the conservation of endangered species. In this regard, *in vitro* techniques utilising both liquid culture (Jha and Sen, 1986) and solid culture (Cooke *et al.*, 1988) of the bulb material have been established for *Bowiea volubilis* (Section 2.1.4). The primary objective of the present investigation aimed to establish non-destructive *in vitro* regeneration protocol(s). In this regard, the adopted strategy involved the use of the inflorescence as explant material. Part of the aim of this investigation was the establishment of a protocol giving high yields and which would be commercially feasible. As a final step in the protocol, the regenerated plantlets were to be hardened-off to be re-introduced into the gene pool.

Table 2.3; Summary of the reported studies on bulbous species

Species	Reference
Allium sp.	DeBergh and Standaert-de-Mesenare (1976); Dunstan and Short (1977); Guha (1966); Havranek and Novak (1976)
Amaranthus ruentas	Flores *et al.* (1981); Gamborg *et al.* (1968)
Amaranthus gangeticus	Flores *et al.* (1981)
Amaranthus hypochondriacus	Flores *et al.* (1981)
Amaryllis sp.	Bapat and Narayanaswamy (1976); Seabrook and Cumming (1976)
Anthurium andreanum	Kunisaki (1980)
Asparagus sp.	Chin (1982); Fonnesbech (1975); Gorter (1965); Kar and Sen (1985); Murashige *et al.* (1972); Steward and Mapes (1971); Yang (1976)
Dioscorea sp.	Asokan *et al.* (1983); Forsyth and van Staden (1982)
Freesia	Brants (1968)
Gladiolus sp.	Bajaj *et al.* (1983); Simonson and Hildebrandt (1971)
Gloxinia sp.	Bigot (1974); Haramaki and Murashige (1972)
Heloniopis sp.	Kato (1974, 1975a, 1975b)
Hippeastrum hybridum	Mii *et al.* (1974)
Lilium sp.	Hackett (1970); Kato and Hasutake (1977); Niimi and Onozawa (1979); Novak and Petru (1981); Robb (1957); Simmonds and Cumming (1976); Stimart and Ascher (1978); Takayama and Misawa (1979); Torres and Natarella (1982)
Laportea sp.	Tanno (1977)
Muscaria meriacum	Peck and Cumming (1986)
Narcissus sp.	Seabrook and Cumming (1978); Steinitz and Yahel (1982)
Nerine sp.	Grootaarts *et al.* (1981); Kim *et al.* (1981); Pierik and Ippel (1977); Pierik and Woets (1971)
Tuberosa	Muralydher and Mehla (1986)
Urginia indica	Jha *et al.* (1984)

Table 2.4: Some of the established protocols for the micropropagation of bulbous species

Species	Explant type	Regeneration route	Medium	PGRs (mg/l)	Results	Author/s
Allium sp.	radicles	embryogenesis	Dunstan and Short (1977)	(0.138) 2,4-D + (0.05) NAA	callus	Dunstan and Short (1977)
	primordial flower head	organogenesis	Dunstan and Short (1977)	(0.05-2) NAA + (2-8) 2iP	shoots	Dunstan and Short (1977)
	twin scales	organogenesis	Hussey (1978)	(4) BAP (shoots)	plantlets	Hussey and Falavigna (1980)
				(0.5) IBA + (0.12) BAP (roots)		
	various	organogenesis	Murashige and Skoog (1962)	(1.75) IAA + (0.2) Kin (callus)	plantlets	Havranek and Novak (1976)
				(1.25) IAA + (2.2) Kin (shoot buds)		
				(0.1-2) NAA (roots)		
	shoot tips	organogenesis	Murashige and Skoog (1962)	(0.5) 2iP	shoots	Thakur *et al.* (1994)
Amaranthus hypochondriacus	hypocotyls	organogenesis	Gamborg *et al.* (1968)	(0.1) NAA + (0.1) Zea	shoots	Flores *et al.* (1981)
Amaryllis spp.	anther/bulb	organogenesis	Bapat and Narayanaswamy (1976)	(2) 2,4-D + (1) Kin (callus)	plantlets	Bapat and Narayanaswamy (1976)
				(0.5) 2,4-D + (1) Kin + 10% CM (shoot)		
				none (bulbs and roots)		
Blandfordia grandiflora	shoot tips	organogenesis	Murashige and Skoog (1962)	(0.5) BAP (shoots)	plantlets	Johnson (1990)
				(1.5) IBA (roots)		
Dioscorea bulbifera	nodal stem segements	organogenesis	Murashige and Skoog (1962)	(1) BAP	shoots	Forsyth and van Staden (1982)
Freesia spp.	anthers	organogenesis	Murashige and Skoog (1962)	(0.5) NAA + (2) PBA + (1) Kin	plants	Bajaj and Pierik (1974)
Galtonia spp.	floral parts	organogenesis	Murashige and Skoog (1962)	(1) NAA + (0.3) BAP (shoot initiation)	plantlets	Drewes and van Staden (1993)
				(0.5) NAA (shoots and roots)		
Gladiolus spp.	inflorescence stem	organogenesis	Bajaj and Pierik (1974)	(10) NAA + (0.5) Kin	callus	Bajaj *et al.* (1982)
	cormel segments	organogenesis	Murashige and Skoog (1962)	(0.1) NAA + (0.5) Kin	plantlets	Bajaj *et al.* (1982)
	nodal buds	organogenesis	Murashige and Skoog (1962)	(5) BAP	shoot buds	Grewel *et al.* (1990)
	shoot tips	organogenesis	Murashige and Skoog (1962)	(4) Kin (shoots)	plantlets	Negi *et al.* (1990)
				(1) IBA (cormels)		
	corm tissue	organogenesis	Murashige and Skoog (1962)	(1) BAP + (0.1) NAA (shoot buds)	plantlets	Prasad (1990)
				(0.25) BAP (shoot mult.)		
				(0.1) NAA (cormlets)		
	inflorescence stalk	organogenesis	Murashige and Skoog (1962)	(10) NAA + (0.5) Kin (shoots and roots)		
				(0.5) Kin (bulbs)		
Gloxinia hybrida	floral pedancles	organogenesis	Bigot (1974)	(0.2) NAA + (1) BAP (buds)	plantlets	Bigot (1974, 1975)
				(0.5) IAA + (50) BAP (shoots)		
Heloniopis orientalis	stem internode/leaves	organogenesis	Murashige and Skoog (1962)	(2.3) BAP (adv. buds)	plantlets	Kato (1975a,b)
				none		
Hippeastrum hybridum	bulb scale	organogenesis	Murashige and Skoog (1962)	(5-10) NAA + (0.2) Kin	shoots, roots and bulbs dep. on ratio	Mii *et al.* (1974)
	bulb scale/basal plate	organogenesis	Heinz and Mee (1969)	(2) NAA (adv. shoots)	shoots	Hussey (1975)
Hyacinthus aenthystina	flower bud			(6) BAP + (0.3) NAA (bulbs)	plantlets	Kim *et al.* (1981)
				(0.1) NAA (roots)		
	bulb scale	organogenesis	Murashige and Skoog (1962)	(0.1-1) NAA + (0.05-5) BAP (bulbs)	plantlets	Vidor and Perez (1994)
				(1) NAA or (0.5) BAP (roots)		
Laporica bulbifera	bud	organogenesis	Tanno (1977)	(0.4) GA_3	shoots	Tanno (1977)
Lilium spp.	bulb scale	organogenesis	Murashige and Skoog (1962)	(1.13) BAP + (1.1) 2,4-D	plantlets	Simmonds and Cumming (1976)
	stem apex	organogenesis	Linsmaier and Skoog (1965)	(2) IAA (callus)	plantlets	Sheridan (1968)
				none (shoots and roots)		
	bulb scale	organogenesis	Murashige and Skoog (1962)	(0.03) NAA	bulblets	Stimart *et al.* (1982)
	nodal cuttings	embryogenesis	Murashige and Skoog (1962)	(0.1) NAA (embryos)	shoots (rooted *ex vitro*)	Saiju *et al.* (1994)
				(0.01) NAA + (0.5) BAP (shoots)		
Muscaria armeniacum	bulb scale	organogenesis	Heinz and Mee (1969)	(2) NAA + (5) BAP	plantlets	Peck and Cumming (1986)
Narcissus spp.	ovary	organogenesis	Heinz and Mee (1969)	(2-8) 2,4-D	shoots	Hussey (1975)
	young flower stalks	organogenesis	Asahira and Hosoki (1977)	(1) NAA + (5) BAP (adv. shoots)	bulbs	Hosoki and Asahawa (1980)
				(0.1) NAA (bulbs)		
Nerine bowdenii	bulb scale	organogenesis	Murashige and Skoog (1962)	(0.1) NAA + (1) BAP	plantlets	Chow *et al.* (1990)
	bulb scale/basal plate	organogenesis	Grootaarts *et al.* (1981)	(1) IBA	shoots	Grootaarts *et al.* (1981)
Schizobasis intricata	bulb scale	organogenesis	Murashige and Skoog (1962)	(2) BAP + (2) NAA (shoots)	plantlets	Drewes *et al.* (1993)
				(1) NAA (roots)		
Thuranthos basuticum	bulb	organogenesis	Murashige and Skoog (1962)	(5) NAA + (1) BAP (shoots)	plantlets	Jones *et al.* (1992)
				(5) NAA + (0.1) BAP (roots)		
Tulipa spp.	bulb scale	organogenesis	Murashige and Skoog (1962)	2,4-D and BAP	shoots	Koster (1990)
Urginia indica	basal disc and scales	organogenesis	Jha *et al.* (1984)	(4) 2,4-D + (2) NAA + (2) Kin (callus)	plantlets	Jha *et al.* (1984)
				(2) 2,4-D + (1) NAA + (2) Kin (shoots)		
				(0.5) NAA + (0.5) Kin (bulbs)		
Watsonia spp.	shoot tips	organogenesis	Murashige and Skoog (1962)	(1) BAP	plantlets	Jona *et al.* (1994)

2.2.MATERIALS AND METHODS

2.2.1. Establishment of aseptic cultures: a preliminary investigation of contamination from the inflorescence stem

Pieces of inflorescence stem (500 mm long) from a mature plant were washed and surface-sterilised. This was carried out by dipping the plant material into 70 % [v/v] ethanol for 2 - 3 minutes, whereupon it was transferred to a solution of 1 % sodium hypochlorite containing 5 drops of Tween-20 for 20 minutes. After sterilisation, the plant material was rinsed three times with sterile water under sterile conditions. Subsequently, 10 mm-long explants were cut from the inflorescence and placed onto MS (Murashige and Skoog, 1962) nutrient medium containing 30 $g.l^{-1}$ sucrose and solidified with 10 $g.l^{-1}$ agar. The pH of the medium was adjusted to between 5.6 and 5.8 prior to autoclaving. The presence of contaminants was recorded after 5 days in culture at 25 °C and 16:8 h light:dark photoperiod (200 $\mu Em^{-2}s^{-1}$).

2.2.2. Generation of explant types from the inflorescence stem

The inflorescence stem of *B. volubilis* is intricately branched (Plate 2.1 and Figure 2.1). As a result of this, several types of explants were identified and produced and their regeneration capacity investigated. The stem was divided into two regions, namely the primary stem and the branches as illustrated in Figure 2.1A.

The primary inflorescence stem was divided into five approximately equal regions along its length. Each region contained ten nodes (from which nodal explants were generated) and internodal regions (internodal explants). The region closest to the bulb

was termed the basal region (from which the basal explants were generated). The division of the primary stem is shown in Figure 2.1B. The branches of the inflorescence stem were divided into three regions from which explants were generated as depicted in Figure 2.1C.

2.2.3. Regeneration of *Bowiea volubilis*

2.2.3.1. Micropropagation using the branches of the inflorescence stem as explants

The explants were prepared as described in Section 2.2.2. Several nutrient media (Dollfus and Nicolas-Prat, 1969; Dunstan and Short 1977; Linsmaier and Skoog, 1965; Margara, 1969; Murashige and Skoog, 1962; Ziv *et al.*, 1970) supplemented with various types, concentrations and combinations of plant growth regulators were tested in order to determine the optimum medium for embyogenesis or organogenesis (Table 2.5). After 6 - 8 weeks on induction media, explants were transferred to the same medium containing no plant growth regulators (PGRs). Regenerated plantlets were excised from the original explant and cultured onto LS (Linsmaier and Skoog, 1965) nutrients devoid of growth regulators, for plantlet growth. All culture media were supplemented with sucrose or glucose, as shown in Table 2.5, and contained 10 $g.l^{-1}$ agar. In cases where pro-embryos were formed, several germination media were tested [LS nutrients supplemented with 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar, and either: (1) 4 $g.l^{-1}$ activated charcoal, (2) 2 $mg.l^{-1}$ IAA and 1 $mg.l^{-1}$ kinetin or (3) a combination of (1) and (2)]. Explants were maintained at 25 °C either in the dark or in the light with a 16:8 hour photoperiod (200 $\mu Em^{-2}s^{-1}$).

2.2.3.2. Micropropagation using the primary inflorescence stem

The basal medium used for the regeneration of *B. volubilis* using explants obtained from the primary inflorescence stem, consisted of MS nutrients supplemented with either 15 $g.l^{-1}$ or 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar and plant growth regulators as shown in Table 2.6. The growth conditions were as those described in 2.2.3.1 above. Pieces of the primary stem were cut transversally to produce explants 1 mm thick (Taeb and Alderson, 1987) and the results were recorded after 10 weeks in culture.

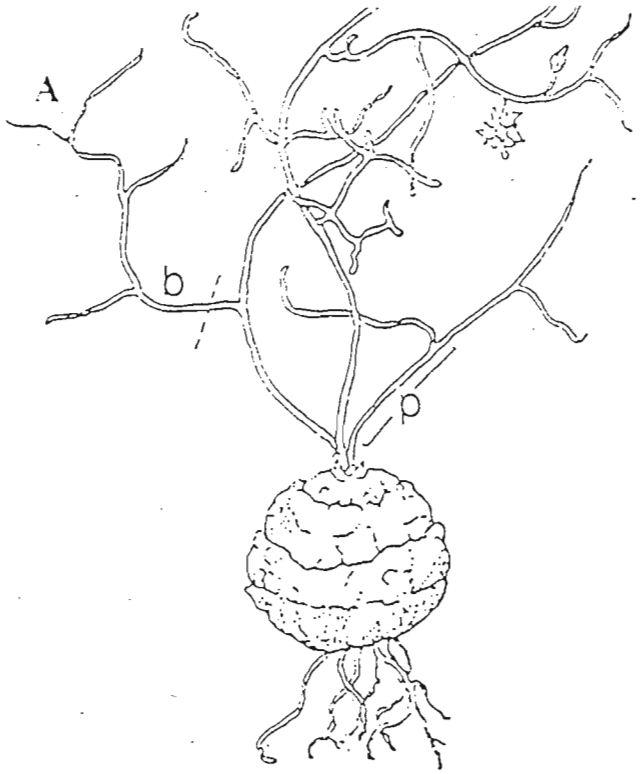
2.2.4. Hardening-off and acclimatisation of plantlets

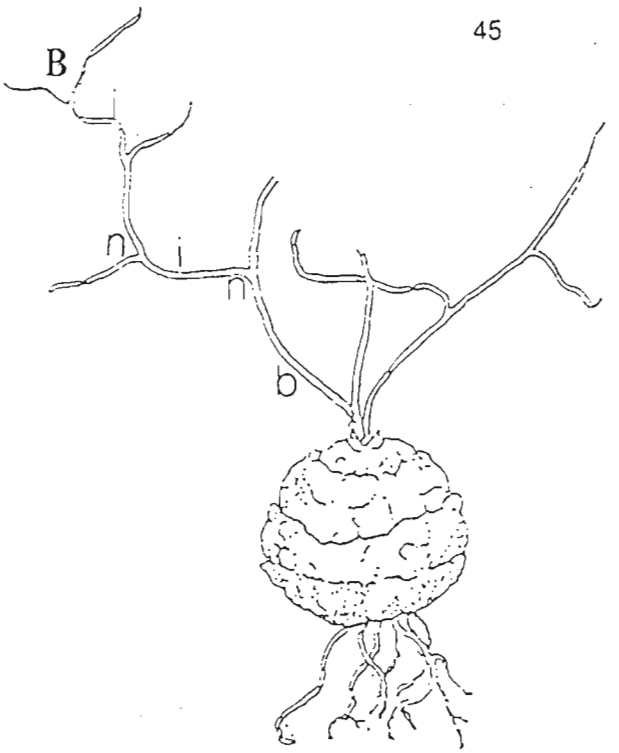
Regenerated plantlets were transplanted into a mixture (1:1) of autoclaved potting soil and washed coarse river sand. Three different methods were used in order to determine the most appropriate way of hardening-off the plantlets in order to maintain a high survival rate *ex vitro.* Plants were either:

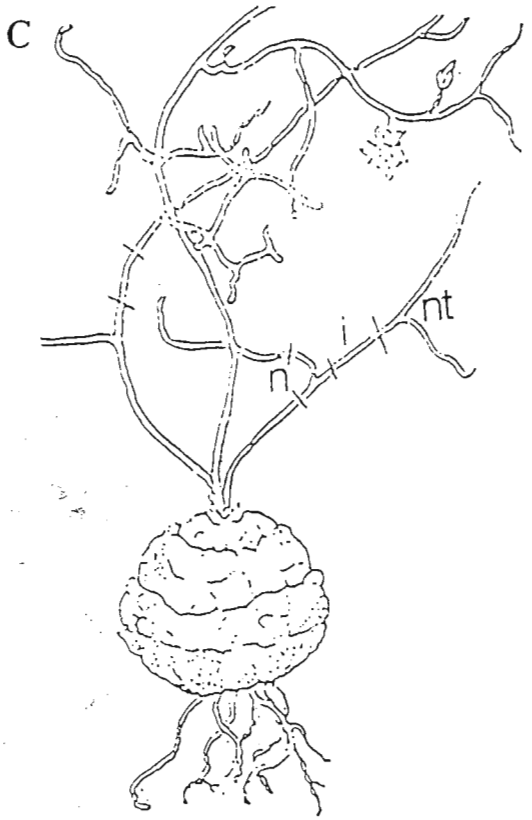
1. tightly covered with plastic bags (high humidity) for 7 days after which the humidity of the microclimate of each plantlet was reduced gradually by opening the bags daily for increasingly longer periods over 2 - 3 weeks, when the bags were removed and the plantlets treated as mature plants; or

2. plantlets were loosely covered with plastic bags (medium humidity) and humidity was retained within the microclimate of each plantlet but a lower humidity compared with that achieved by the method described above, was maintained; or

3. plants were not covered at all, but were mist-sprayed twice daily with tap water (low humidity), ensuring that the soil:sand mixture was kept fairly moist.







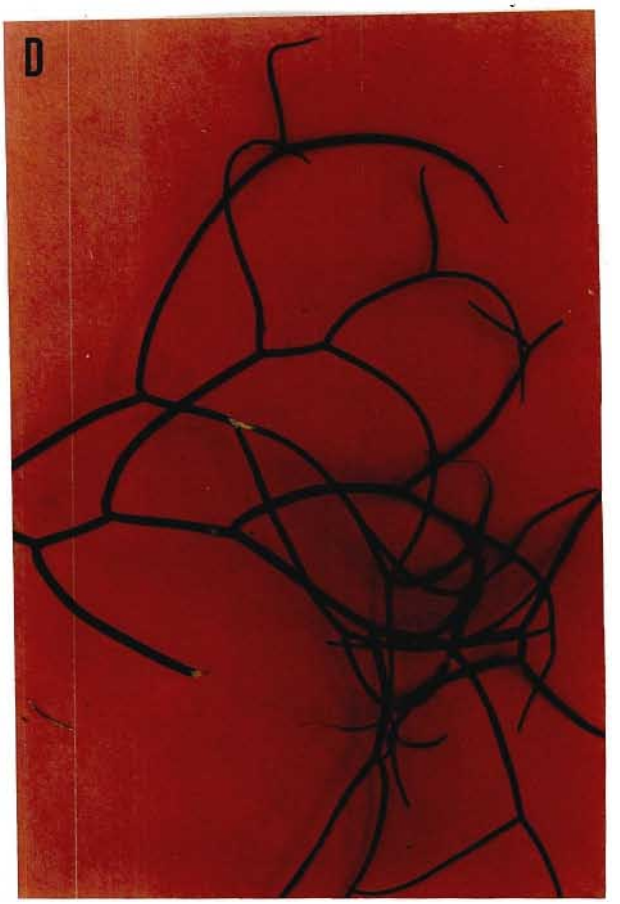


Figure 2.1: Diagrammatic representation of the selection and sampling of different types of explants from the inflorescence stem of *B. volubilis*

A: A representation of the growth form of *B. volubilis*. p = primary stem; b = branches of inflorescence stem
B: The generation of explants from the primary stem. b = basal explant; n = nodal explant; i = internodal explant
C: The generation of explants from the branches of the inflorescence stem. nt = nodal tip; n = nodal; i = internodal
D: Piece of the inflorescence stem showing explants responding to treatments. n = nodal; i = internodal

Table 2.5: Composition of media and light treatments tested for culture response (induction of embryogenesis or organogenesis) using explants derived from the branches of the inflorescence stem.

Explants were sampled as shown in Figure 2.2. Cultures that were placed in the light underwent a 16:8 h light/dark photoperiod with light intensity of 200$\mu Em^{-2}s^{-1}$. PGRs = plant growth regulators.

Medium	PGR ($mg.l^{-1}$)	Sucrose ($g.l^{-1}$)	Light Treatment
Murashige and Skoog (1962)	(1) NAA + (1) BAP	30	light
Linsmaier and Skoog (1965)	(1) 2,4-D	30	dark
	(3) 2,4-D	30	dark
	(5) 2,4-D	30	dark
	(1) 2,4-D + (0.2) Kin	30	dark
	(3) 2,4-D + (0.2) Kin	30	dark
	(5) 2,4-D + (0.2) Kin	30	dark
	(1) 2,4-D + (0.5) Kin	30	dark
	(3) 2,4-D + (0.5) Kin	30	dark
	(5) 2,4-D + (0.5) Kin	30	dark
	(1) 2,4-D + (1) BAP + (0.2) Kin	30	dark
	(0) NAA + (10) BAP	30	light
	(0.3) NAA + (3) BAP	30	light
	(9.3) NAA + (1.1) BAP	30	light
	(9.3) NAA + (0.1) BAP	30	light
	(18) NAA + (1.1) BAP	30	light
	(20) NAA + (0.1) BAP	30	light
	(1) NAA + (1) BAP	30	light
Margara (1969)	(2) BAP	45	light
Dollfus and Nicolas-Prat (1969)	(0.1) IAA + (1) Kin + (10) $AdSO_4$	10 (glucose)	light
Ziv *et al.* (1970)	(10) NAA + (0.5) Kin + (160) $AdSO_4$	30	light
Dunstan and Short (1977)	(1) BAP	30	light

Table 2.6: Different combinations of plant growth regulators (PGRs) tested on each type of explant on the primary inflorescence stem. Explants were sampled as shown in Figure 2.2. Culture medium contained MS nutrients supplemented with plant growth regulators (PGRs) and 10 $g.l^{-1}$ agar. CH = casein hydrolysate ($mg.l^{-1}$).

Explant	PGRs	Sucrose ($g.l^{-1}$)	Other constituents ($mg.l^{-1}$)	Light Treatment
nodal and basal	(1) BAP + (1) NAA	30	CH 500	light
nodal and basal	(0.5) BAP + (1) NAA	30	CH 500	light
nodal and basal	(1) BAP + (0.5) NAA	30	CH 500	light
internodal	(1) 2,4-D	15	-	dark
internodal	(1) 2,4-D	30	-	dark
internodal	(3) 2,4-D	15	-	dark
internodal	(3) 2,4-D	30	-	dark
internodal	(1) 2,4-D + (0.1) Kin	30	-	dark
internodal	(1) 2,4-D	30	CH 500	dark
internodal	(3) 2,4-D	30	CH 500	dark

2.2.5. Photography

All stages of development were recorded using a Nikon FM2 camera with a 60 mm Mikro Nikkor macro lens.

2.2.6. Data Analysis

Average values were calculated from the data recorded during the different stages of plant regeneration. The response of explants to treatments was averaged and percentages calculated for each treatment. These percentages were used in the statistical analysis (pseudoreplication). Where appropriate, one-way ANOVA

(Statgraphics Plus, 1993) was used to assess differences in the recorded mean values of the variables investigated (P = 0.05). Alphabetical values were assigned to the mean values recorded for each treatment. Mean values that did not share the same letter, were recognised as being significantly different from each other.

2.3. RESULTS

2.3.1. Production of sterile explants from field grown material

A preliminary study was performed to determine the extent of contaminants in *B. volubilis* cultures. After 5 days in culture, neither fungal nor bacterial contaminants were present in any of the cultures, indicating that the surface sterilisation protocol employed was adequate.

2.3.2. Micropropagation of *Bowiea volubilis*

2.3.2.1. Response of the inflorescence stem to applied treatments

Irrespective of the medium used (Section 2.2.3.2), explants obtained from the primary inflorescence stem (Figure 2.1) showed no response after 10 weeks in culture. In contrast, a visual assessment after 2 - 3 weeks, showed that internodal and nodal explants from the branches (Figure 2.1) exhibited regenerative potential (Figure 2.2). Consequently, only nodal and internodal explants from the branches were used in subsequent studies.

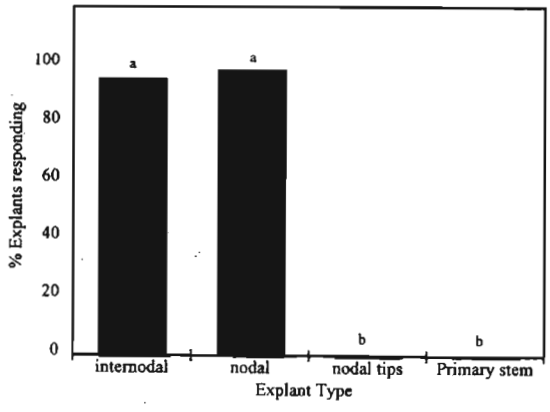


Figure 2.2: Effect of explant source on initial response to culture medium. (Explants sampled as described in Section 2.2.2, Figure 2.2) n (number of explants) = 100-120.

2.3.2.2.Organogenesis in *Bowiea volubilis*

a) The process

In this investigation, induction of organogenesis was attempted on LS and MS nutrient media supplemented with combinations of growth hormones (Table 2.5). With the best hormone combination for induction of plantlet regeneration (1 $mg.l^{-1}$ 2,4-D and 1 $mg.l^{-1}$ BAP), swellings developed [after 2 - 4 weeks in the dark (Plate 2.2A)] near the cut ends of internodal explants and near the cut ends and at the junction of branches of nodal segments. After 6 - 8 weeks in culture, bulb-like structures developing from the swellings were observed on explants (Plate 2.2B). After 8 - 10 weeks, the explants were transferred to the light and cultured onto a second medium which was devoid of growth hormones. Shoot extension from the bulblets was observed (Plate 2.2C) within 2 - 3 weeks. Root emergence occurred 1 - 2 weeks after shoot extension (Plate 2.2D) and subsequently plantlets were excised from the original explant and transferred to culture bottles for growth (Plate 2.2E). The plantlets were hardened-off (Plate 2.2F) after 2 - 3 weeks as described in Section 2.2.4. After 4 - 5 months, regenerated plants kept in the greenhouse, produced inflorescence stems (Plate 2.2G).

b) The effect of medium composition on organogenesis

Organogenesis in *B. volubilis* was induced on nutrient media supplemented with different combinations of BAP and the auxins 2,4-D and NAA (Table 2.7). Some treatments resulted in organogenically-derived plantlets. The best results resulted in plantlet regeneration on LS nutrients supplemented with 1 $mg.l^{-1}$ 2,4-D and 1 $mg.l^{-1}$ BAP, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar (Table 2.7). 77.9 % of explants exhibited direct

formation of bulb-like structures after 6 - 8 weeks in culture (Table 2.7). Different combinations of BAP and NAA were tested for the induction of organogenesis on explants (Table 2.7). The degree of organogenesis ranged from 0 - 85.2% (Table 2.7) for the different treatments. Bulblet development was observed after 10 - 12 weeks in culture, on explants maintained on 1 $mg.l^{-1}$ BAP and 1 $mg.l^{-1}$ NAA (Plate 2.2B) whereas explants on medium supplemented with 10$mg.l^{-1}$ BAP only, became necrotic and died after 2 - 3 weeks. Explants on medium with 9.3 $mg.l^{-1}$ NAA and 1.1 $mg.l^{-1}$ BAP developed bulblets in the same time: however, the higher concentration of NAA appeared to inhibit shoot and root development.

Significantly higher ratios of NAA:BAP resulted in bulblet formation at a lower rate (Table 2.7) than treatments using lower ratios of these plant growth regulators. Explants on media supplemented with 9.3 $mg.l^{-1}$ NAA and 0.1 $mg.l^{-1}$ BAP and 20 $mg.l^{-1}$ NAA and 0.1 $mg.l^{-1}$ BAP developed prolific protrusion of roots after 14 - 16 weeks in culture (Plate 2.3).

The addition of 0.2 $mg.l^{-1}$ kinetin to the medium resulted in a decrease in the number of explants producing organogenic structures (35.7 %, Table 2.7) and a delay in bulblet formation (from 8 - 10 weeks to 14 - 16 weeks) compared with treatments not containing kinetin. Furthermore, no shoot elongation was observed in the presence of kinetin.

Medium formulation appeared to be important in the development of organs. Explants on Murashige and Skoog (1962) medium and the same growth regulator

combination produced bulblets and root extension. However, none of the explants produced shoots. The nutrient media of Dollfus and Nicolas-Prat (1969), Dunstan and Short (1977), Margara (1969) and Ziv *et al.* (1969) did not induce swellings (as described in Sections 2.3.2.2 and 2.3.2.3) in any explants, rather, their use was associated with necrosis and death after 2 - 3 weeks.

Table 2.7: The effect of growth regulators on organogenesis of *Bowiea volubilis*. Explants were cultured onto LS nutrient medium supplemented with 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. Plant organs are denoted: (B) = bulblets only, (P) = plantlets, (R) = roots only. n (number of explants) = 80-100 per treatment.

Medium		% Explants that exhibited a response	Number of plantlets/explant
Nutrient formulation	PGRs ($mg.l^{-1}$)		
Linsmaier and Skoog (1965)	(1) 2,4-D + (1) BAP	77.9 (P)^{h}	4.6
	(1) 2,4-D + (1) BAP + (0.2) Kin	35.7 (B)^{c}	-
	(9.3) NAA + (1.1) BAP	85.2 (B)^{i}	-
	(9.3) NAA + (0.1) BAP	76.3 (R)^{g}	-
	(18) NAA + (1.1) BAP	16.1 (R)^{b}	-
	(20) NAA + (0.1) BAP	48.7 (R)^{e}	-
	(1) NAA + (1) BAP	68.5 (P)^{f}	2.1
	(0) NAA + (10) BAP	0.0^{a}	-
Murashige and Skoog (1962)	(1) NAA + (1) BAP	43.2 (R)^{d}	-

c) Evaluation of protocols for organogenesis with respect to plantlet regeneration

The optimum regeneration media for the production of plantlets were found to be LS nutrient medium supplemented with 1 $mg.l^{-1}$ NAA and 1$mg.l^{-1}$ BAP or 1$mg.l^{-1}$ 2,4-D and 1 $mg.l^{-1}$ BAP as described above (Section 2.3.), although the rate of plantlet

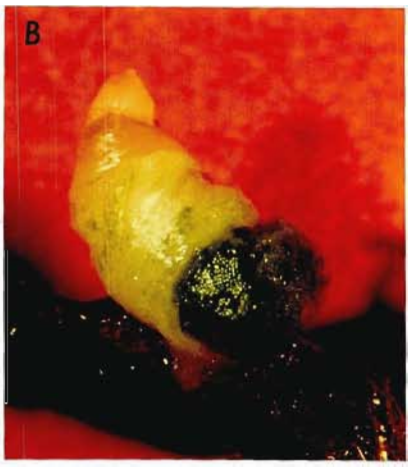
production was slower and lower for the former treatment. An average of 4.6 plantlets could be regenerated from a single explant using the best treatment whereas an average of 2.1 plantlets were regenerated per explant using 1 $mg.l^{-1}$ BAP and 1 $mg.l^{-1}$ NAA (Table 2.7).

2.3.2.3. Somatic embryogenesis in *Bowiea volubilis*

a) The process

Another strategy attempted to mass propagate this species, was via somatic embryogenesis. Explants were cultured onto LS nutrient media containing 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar and several plant growth regulator combinations (Table 2.5) and after 2 - 4 weeks, the development of swellings (Plate 2.4A) was observed (as described in Section 2.3.2.2). Of the media tested, the best hormone combination for the induction of pro-embryos was 5 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ kinetin. This combination resulted in 72.7 % of explants developing pro-embryos. The pro-embryos (Plate 2.4B and 2.4C) appeared to grow from within the swellings on the explants with little callus formation (i.e. direct embryogenesis). After 10 - 12 weeks in culture, microscopical examination revealed that globular- (Plate 2.4D) and heart-shaped (Plate 2.4E) embryos had developed. Embryo maturation did not occur upon transfer to a medium without plant growth regulators. Several germination media i.e. LS nutrients supplemented with 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar and either: (1) 4 $g.l^{-1}$ activated charcoal, (2) 2 $mg.l^{-1}$ IAA and 1 $mg.l^{-1}$ Kinetin, or (3) a combination of the previous treatments were tested for embryo maturation and/or germination. To date (10 weeks) none of the embryos germinated on any of the media tested.


A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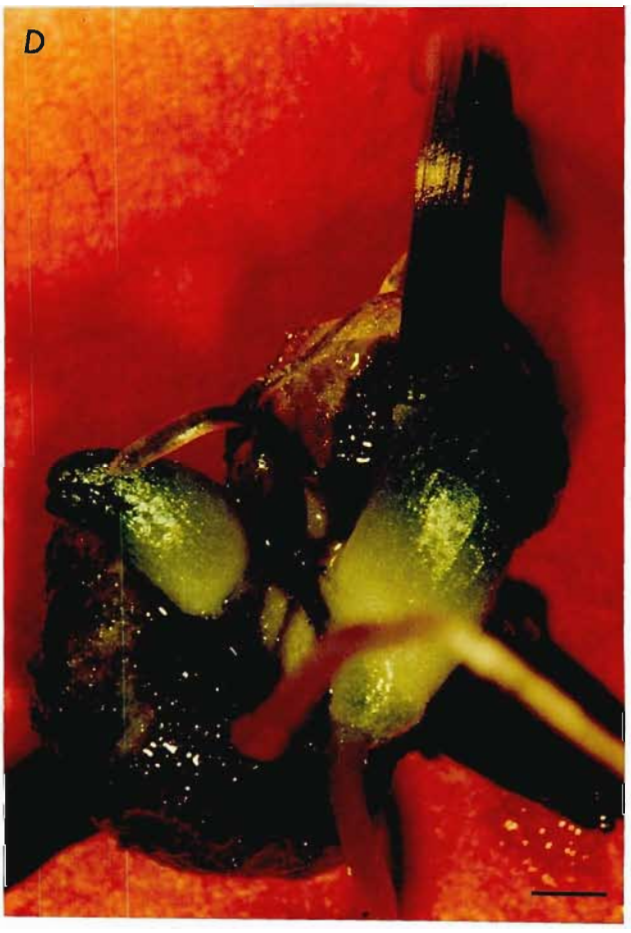
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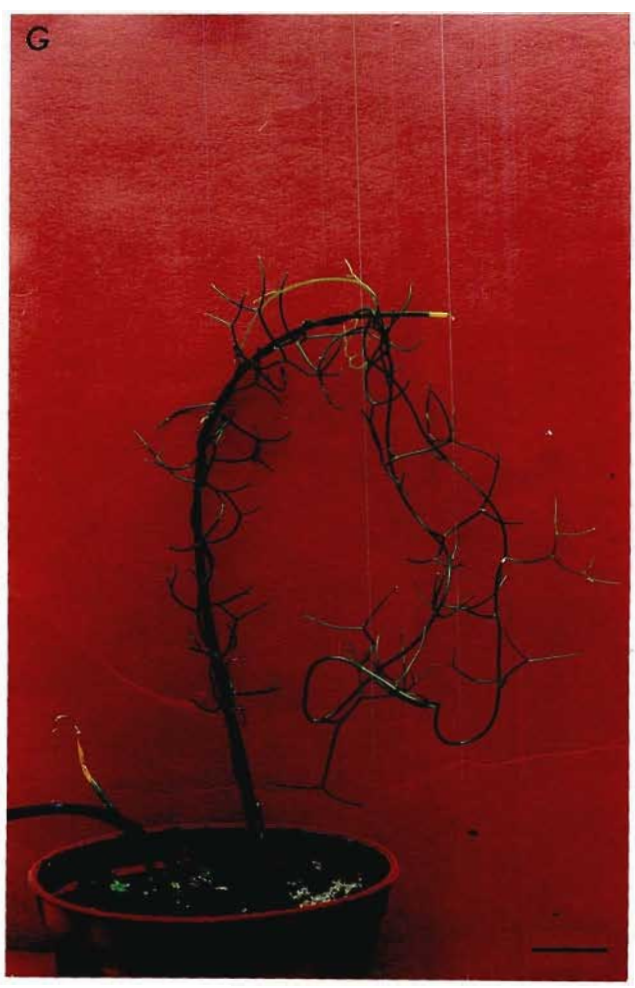
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b) The effect of medium composition on the induction of somatic embryogenesis

Induction of somatic embryogenesis in *B. volubilis* was found to require particular combinations of both auxins and cytokinins (Table 2.8). LS nutrient medium supplemented with varying concentrations of 2,4-D (1, 3 and 5 $mg.l^{-1}$) did not initiate a response in any of the explants. However, the same media supplemented with 0.2 $mg.l^{-1}$ kinetin, induced pro-embryo formation (Table 2.8). Explants on medium containing 1 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ kinetin developed callus after 10 - 12 weeks in culture. After 14 - 16 weeks in culture, however, large abnormal structures had developed (Plate 2.5A and 2.5B). In contrast, explants on medium supplemented with the higher concentrations of 2,4-D (3 and 5 $mg.l^{-1}$) had well developed embryonic structures (Plate 2.4D and 2.4E) after the same time period. However, after 14 - 16 weeks, explants on 3 $mg.l^{-1}$ 2,4-D exhibited extensive root growth whereas globular and heart-shaped embryonic structures could be observed on explants maintained on medium containing 5 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ kinetin. More mature embryo structures were not observed at later stages in culture. The addition of 0.2 $mg.l^{-1}$ kinetin appeared to have a marked effect on somatic embryogenesis in comparison to medium supplemented with 2,4-D only. A medium containing a higher concentration (0.5 $mg.l^{-1}$) of kinetin was tested. Embryo development was significantly reduced (to 19 - 21.4%) when explants were placed on medium containing a high concentration of kinetin as compared with the medium containing 0.2 $mg.l^{-1}$ kinetin (56.1 - 72.7%). In addition, embryos could be observed after only 14 - 16 weeks i.e. embryo development was delayed in these cultures. After 20 weeks in culture root development was observed (Plate 2.3).

Besides using 2,4-D to induce embryogenesis, combinations of NAA and BAP have been used to induce regeneration in many plants. In this study, the best combination for embryo induction was LS nutrients supplemented with 0.3 $mg.l^{-1}$ NAA and 3 $mg.l^{-1}$ BAP. Embryo clumps were observed after 14 - 16 weeks in culture (Plate 2.4B) but no embryo maturation occurred.

The nutrient media of Dollfus and Nicolas-Prat (1969), Dunstan and Short (1977), Margara (1969) and Ziv *et al.* (1970) did not induce swellings in any explants and the plant tissue became necrotic and died after 2 - 3 weeks in culture which is similar to the situation described for organogenesis (Section 2.3.2.2).

Table 2.8: The effect of plant growth regulators (PGRs) on somatic embryogenesis in *Bowiea volubilis* (6 - 9 weeks in culture). Explants were cultured onto LS nutrient medium supplemented with 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. n (number of explants) = 80-100 per treatment.

PGRs ($mg.l^{-1}$)	% Explants undergoing embryogensis
(1) 2,4-D + (0.2) Kin	63.1^{e}
(3) 2,4-D + (0.2) Kin	56.1^{d}
(5) 2,4-D + (0.2) Kin	72.7^{f}
(1) 2,4-D + (0.5) Kin	19.0^{a}
(5) 2,4-D + (0.5) Kin	21.4^{b}
(0.3) NAA + (3) BAP	24.4^{c}

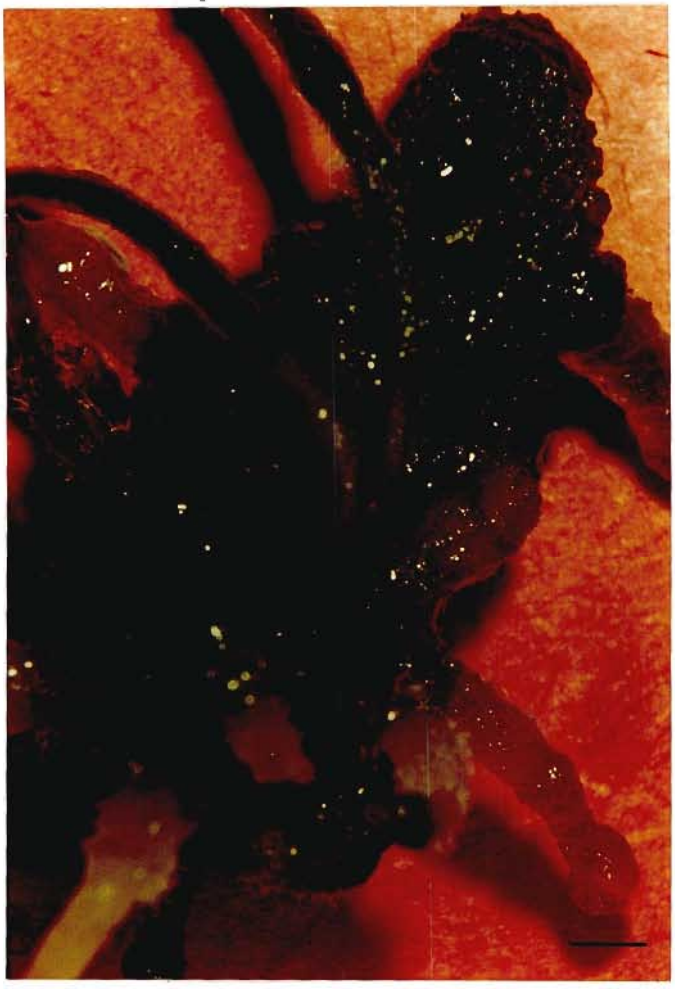


Plate 2.3: Prolific root protrusion from explants after 14-16 weeks on LS nutrients. Explants were initially cultured on medium containing 9.3 mg.l^{-1} 2,4-D and 0.1 mg.l^{-1} BAP, 20 mg.l^{-1} NAA and 0.1 mg.l^{-1} BAP, 1 mg.l^{-1} 2,4-D and 0.5 mg.l^{-1} kinetin or 3 mg.l^{-1} 2,4-D and 0.5 mg.l^{-1} kinetin. Culture medium was supplemented with 30 g.l^{-1} sucrose and 10 g.l^{-1} agar. (bar = 0.8 mm)

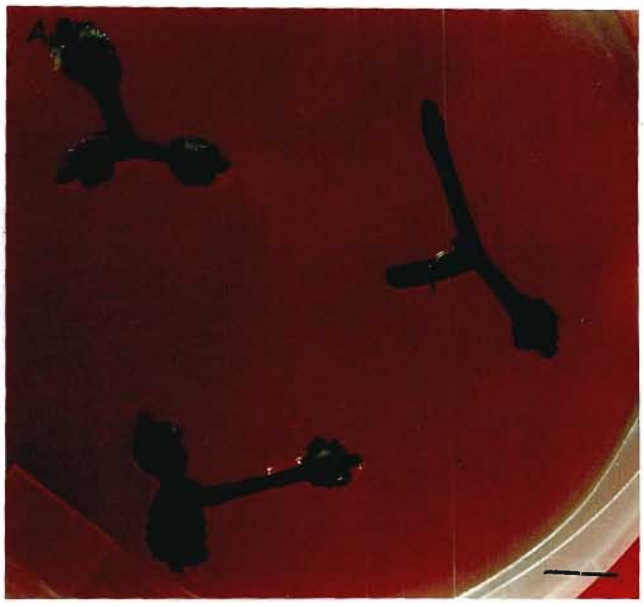
Plate 2.4: Induction of somatic embryogenesis in *Bowiea volubilis*. Explants were cultured onto LS nutrient medium supplemented with 1 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ kinetin, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar.

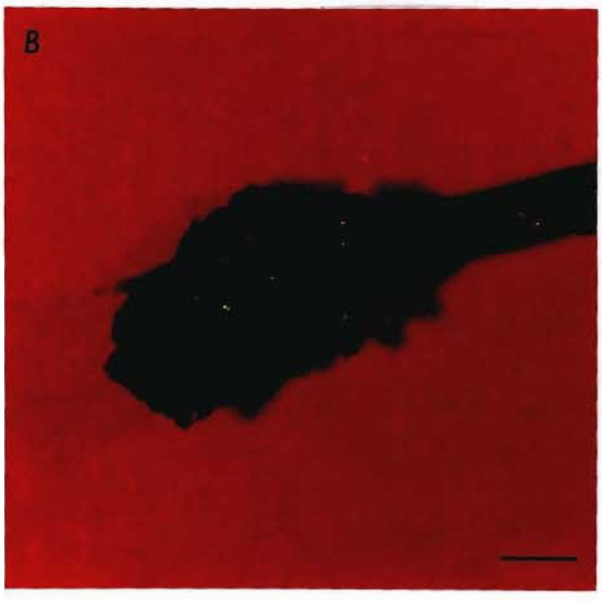
A. The development of swellings on nodal and internodal explants after 6 - 9 weeks in culture. (bar = 20 mm)

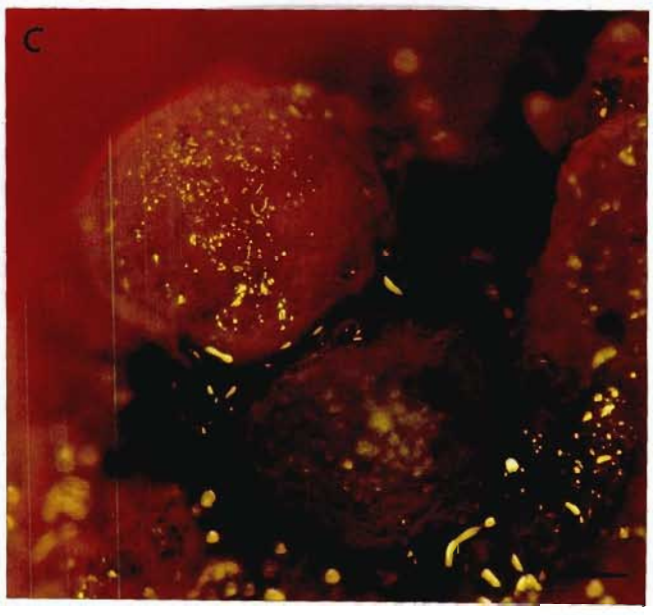
B & C. Embryoid development on swelling near the cut end of the explant after 10 weeks in culture. (B: bar = 1 mm; C: bar = 0.09 mm)

D. Globular embryos dissected out after 10-12 weeks in culture. (bar = 2 mm)

E. Well-developed heart-shaped embryos dissected out after 10-12 weeks in culture. (bar = 2 mm)


A


B


C


D


E

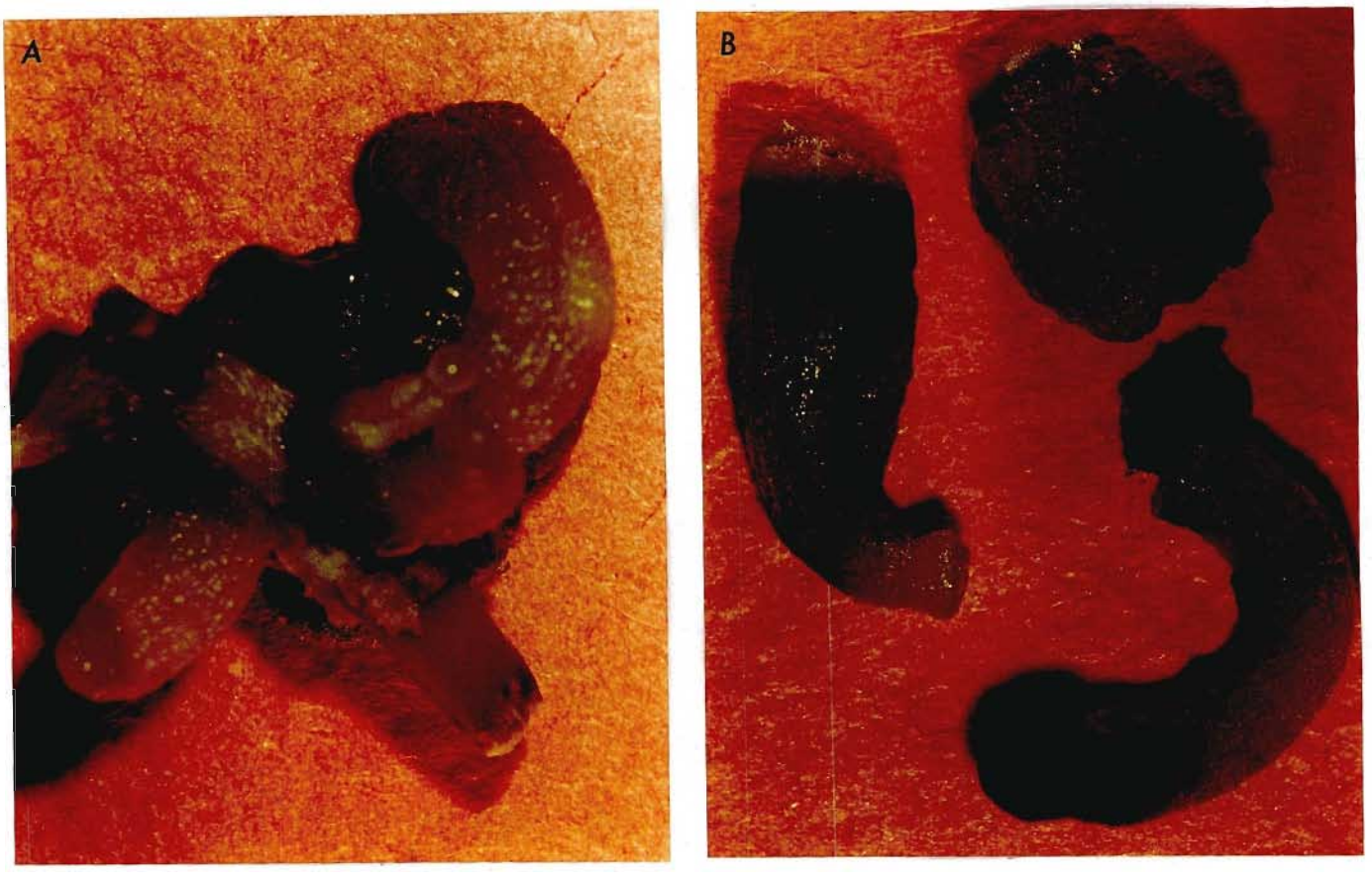


Plate 2.5: Large abnormal structures produced by explants cultured on Linsmaier and Skoog (1965) nutrient medium supplemented with 0.2 mg.l^{-1} kinetin and 1 mg.l^{-1} 2,4-D after 14 - 16 weeks in culture. Culture medium was supplemented with 30 g.l^{-1} sucrose and 10 g.l^{-1} agar

A. Direct formation of abnormal structures with no intermediate callus production. (bar = 1.2 mm)

B. Individual abnormal structures after removal from the explant. (Scale bar = 1.5 mm)

2.3.3. Acclimation of *in vitro* regenerated plantlets to ambient conditions

2.3.3.1.The effect of humidity on plantlet survival

Plantlets regenerated in culture were hardened-off according to the methods described in Section 2.2.4. It was found that plantlets required a microclimate with a low relative humidity in order to ensure a high survival rate *ex vitro* (Table 2.9). A twice-daily mist spray appeared to be the most effective hardening off treatment (90.9% survival) for regenerated plantlets. Plants maintained at this low level of humidity became established 2 - 3 weeks after planting out, whereafter they were treated as mature plants.

Table 2.9: The effect of acclimatisation conditions on the survival of regenerated plantlets. n (number of plantlets) =30 per treatment.

Hardening-off treatment	% survival of regenerated plantlets
Tightly sealed plastic bags (high humidity)	0.0^{a}
Loosely covered plantlets (medium humidity)	10.2^{b}
Twice-daily mist spray (low humidity)	90.9^{c}

Plantlets tightly sealed in plastic bags and therefore maintained in a microclimate with a high humidity had a 0% survival rate. They either rotted due to excess moisture, or were highly susceptible to fungal attack (the high humidity presumably encouraging rapid germination of fungal spores) resulting in plantlet death 5 - 7 days after planting out.

2.3.3.2. The effect of bulblet size on *ex vitro* plantlet survival

In addition to the effect of humidity on plantlet survival (Table 2.9), it was found that bulblet diameter played an important role in the survival of plantlets after transfer to the potting medium (Table 2.10). Plantlets having bulblets smaller than 4 mm in diameter died 5 - 7 days after planting out whereas larger bulbs survived (Table 2.10).

Table 2.10: The effect of bulblet size on *ex vitro* survival of plantlets (results given three weeks after planting out) n (number of plantlets) = 30 per treatment. Plantlets were planted out into a 1:1 mixture of potting soil and coarse river sand and were mist-sprayed twice-daily with tap water.

Diameter of bulblet (mm)	Survival rate (%)
≤4.0	10.7[a]
>4.0	95.9[b]

2.4. DISCUSSION

2.4.1. Development of micropropagation protocols for *Bowiea volubilis*

Various combinations of plant growth regulators initiated different responses in combination with nutrient media as shown in Tables 2.7 and 2.8. However, plantlets could be regenerated by organogenesis only. None of the media which induced pro-embryos facilitated embryo maturation and subsequent plantlet germination (Section 2.3.2.3). The plantlets obtained via organogenesis were readily hardened-off after 2 - 3 weeks and within 4 - 6 months of hardening-off, these plantlets produced inflorescences (bulb diameter approximately 20 mm). The developed protocol is shown in Figure 2.3 below.

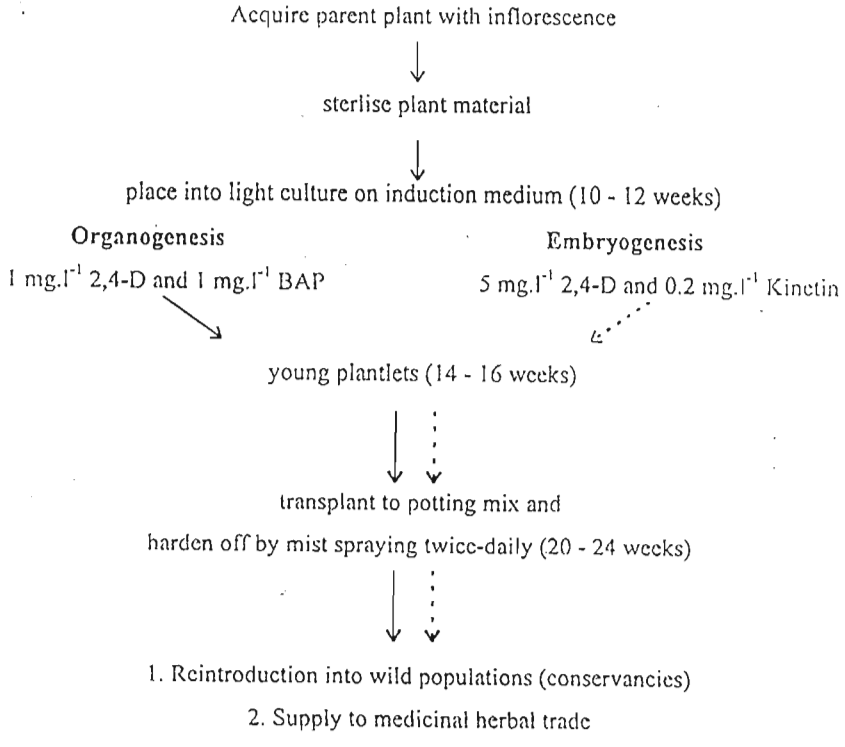


Figure 2.3: Schematic representation of regeneration of *Bowiea volubilis* using the inflorescence stem
(——→ represents successfully developed protocol; - - - - - → represents stages not yet achieved)

It is clear that a large number of plants may be regenerated via organogenesis using this protocol and an estimate of the number of plants which can be generated from the inflorescence of a single parent plant is given in Table 2.11 below.

Table 2.11: Estimate of the number of *Bowiea volubilis* plantlets which may be regenerated from the inflorescence of a single parent plant. Explants cultured on 1 $mg.l^{-1}$ 2,4-D and 1 $mg.l^{-1}$ BAP, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar.

Total inflorescence length (including branches):	± 20m - 30m
Number of 10 mm explants generated:	20 000 - 30 000
Number of explants producing plantlets (77.9 % of total):	15 580 - 23 370
Number of plantlets regenerated (4.6 plantlets/explant):	71 668 - 107 502

In contrast to regeneration of plantlets via organogenesis, regeneration via somatic embryogenesis has the potential to produce even greater numbers of plantlets since each cell has the potential to become embryogenic under appropriate conditions. Future research may result in the establishment of an appropriate embryo maturation medium so that even higher yields of plantlets could be produced through tissue culture.

2.4.2. The effect of explant origin on regeneration capacity

It has been widely reported that the origin of the explant tissue plays an important role in the regenerative capacity of the tissue within the culture system (Cohen, 1986). In the present study, the inflorescence was used as explant material in order to induce

plantlet regeneration without sacrificing the parent plant. The explants responding to any of the treatments (Table 2.5) tested were those that were obtained from the branches of the inflorescence stem (Figure 2.1C and 2.1D). The nodal and internodal explants had a higher regenerative potential than the nodal tip explants also obtained from the branches. In contrast, the explants from the primary stem (Figure 2.1B) appeared to have no regenerative potential under the conditions tested in this study (Table 2.6) which may be attributed to the age of the tissue. Several workers have reported that tissue age plays a vital role in the regenerative capacity of tissues (Wright and Alderson, 1981; Cohen, 1986).

Although the inflorescence stem was used in this study, the most commonly used material for micropropagation of bulbous species, for example, *Hippeastrum hybridum* (Mii *et al.*, 1974), Hyacinth (Hussey, 1975), *Amaryllis* spp. (Bapat and Narayanaswamy, 1976), *Lilium hybrida* (Simmonds and Cumming, 1976), *Allium cepa* (Hussey and Falavigna, 1980), *Nerine bowdenii* (Grootaarts *et al.*, 1981), *Gladiolus* spp. (Bajaj *et al.*, 1983), *Lilium longiflorum* (Stimart *et al.*, 1983), *Urginea indica* (Jha *et al.*, 1984), *Muscari armeniacum* (Peck and Cumming, 1986) *Hyacinth* (Chow *et al.*, 1990; Koster, 1990), *Tulipa* spp. (Prasad *et al.*, 1990; Vidor and Perez, 1994) is bulb scale tissue where, in the majority of studies, the parent plant is sacrificed. Other tissues such as anthers (*Amaryllis* spp., Bapat and Narayanaswamy, 1976), ovaries (Hyacinth, Hussey, 1975), floral stems (*Narcissus*, Hosoki and Asahawa, 1980; *Gladiolus* spp., Bajaj *et al.*, 1982), leaves (*Heloniopis orientalis*, Kato, 1975a,b) and roots (*Allium* spp., Dunstan and Short, 1977) have also been used. Very few workers have reported using the floral stem which has been suggested

(Wright and Alderson, 1981) to be more responsive to shoot regeneration than any other bulb tissues. As mentioned in 2.1.4, *B. volubilis* is extremely vulnerable to over-exploitation so using the inflorescence stem was more desirable than the methods used by Jha and Sen (1986) and Cooke *et al.* (1988) for this species.

2.4.3. The effect of medium formulation

Success in the technology and application of *in vitro* culture techniques is largely due to the achievement of the appropriate nutritional requirements of cultured cells and tissues (Murashige, 1974). The nutrient composition consists of essential nutrients such as inorganic salts, microelements, carbon source and phytohormones. A comparison between the nutrient formulations found to be successful for various bulbous species (Table 2.4) reveals that the majority of researchers have used the high salt media formulations of Murashige and Skoog (1962) or Linsmaier and Skoog (1965). In this study, Linsmaier and Skoog (1965) nutrients (together with the appropriate phytohormones) were found to be successful in the induction of embryogenesis and organogenesis and for regeneration of plantlets via organogenesis from the inflorescence stem. The nutrient formulations of Margara (1969), Dollfus and Nicolas-Prat (1969), Ziv *et al.* (1969) and Dunstan and Short (1977) have all been used in the regeneration of other species (*Allium* spp., *Gladiolus* spp.), using floral stems. However, in the present investigation, explants cultured on these media did not show an initial response to the treatments (i.e. the development of swellings) and furthermore, after 2 - 4 weeks in culture, the explants became necrotic and died.

2.4.4. A comparison of the effect of plant growth regulators on organ regeneration in *B. volubilis* and other bulbous species

A wide range of plant growth regulators have been used to initiate cultures for various bulbous species (illustrated in Table 2.4). Specific combinations of cytokinins and auxins induce the production of callus, shoots, roots and bulbs depending on both the plant tissue used and the species under study. The ratio of a particular auxin (i.e. 2,4-D, NAA or IAA) to cytokinin (KIN and BAP) is important in the establishment of a particular culture (Warren, 1991). High auxin concentrations have been reported to initiate callus formation (Bapat and Narayanaswamy, 1976; Dunstan and Short, 1977; Bajaj *et al.*, 1982; Jha *et al.*, 1984) whereas high cytokinin concentrations initiate shoot production (Bigot, 1974; Bapat and Narayanaswamy, 1976; Dunstan and Short, 1977; Forsyth and van Staden, 1982; Grewel *et al.*, 1990; Johnson, 1990; Negi *et al.*, 1990). In some instances a single growth regulator has been found to induce a particular response such as shoot production (Negi *et al.*, 1990). In a previous study of the micropropagation of *Bowiea volubilis* (Cooke *et al.*, 1988), the optimum hormone combination for shoot regeneration (using bulb scale material) was found to be 1 $mg.l^{-1}$ NAA and 1 $mg.l^{-1}$ BAP after which the shoots were transferred to a hormone-free medium prior to hardening-off. In the present study, using the inflorescence material, the optimum shoot regeneration hormone combinations were found to be 1 $mg.l^{-1}$ 2,4-D and 1 $mg.l^{-1}$ BAP and 1 $mg.l^{-1}$ NAA and 1 $mg.l^{-1}$ BAP (Table 2.7). Similar results were obtained by Pierik and Steegmans (1975) (*Freesia* spp.); Seabrook *et al.* (1975) (*Amaryllis* spp.) and Takayama and Misawa (1979, 1982) (*Lilium* spp.) using bulb-scales.

The concentration of NAA has been found to be an important factor in organogenesis of bulbous species (Alderson and Rice, 1986). However, in this investigation, high ratios of NAA:BAP were found to stimulate prolific root production. Similar results were obtained by Seabrook and Cumming, 1976 (*Hippeastrum* spp.); Takayama and Misawa, 1982 (*Lilum* spp.) and Peck and Cumming, 1986 (*Muscari armeniacum*). From the results obtained in this study and the work of others, it is therefore apparent that the ratio of auxin:cytokinin is critical for bulb induction and subsequent shoot and root extension.

The protocol developed in this study induced bulblet formation prior to shoot and root extension and bulblets formed shoots and roots spontaneously on a nutrient medium without growth regulators. This is in contrast to other species (*Amaryllis* spp., *Narcissus* spp., *Urginea indica*, *Muscaria armeniacum*, *Gladiolus* spp.) where bulblets could be induced only subsequent to shoot and root elongation (Bapat and Narayanaswamy, 1976; Hosoki and Asahawa, 1980; Jha *et al.*, 1984; Peck and Cumming, 1986; Prasad *et al.*, 1990). Furthermore, a specific medium composition was required for bulblet induction in these species. It is important to note that regeneration of *Bowiea* by organogenesis requires only two media, i.e. the induction medium and the hormone-free nutrient medium required for shoot elongation, rooting and plantlet growth. The related species *Urginia indica* and *Thuranthos basuticum* and several other bulbous species (see above) require several more complicated combinations of growth hormones (Table 2.4) to achieve plantlet regeneration.

2.4.5. The application of the developed protocol to conservation strategies

With the presently developed protocol, only two different media are required for plantlet regeneration from the inflorescence stem (initiation of bulblet formation and extension of roots and shoots). The medium composition (LS nutrients with 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar, 1 $mg.l^{-1}$ 2,4-D and 1 $mg.l^{-1}$ BAP) found to be appropriate for plantlet regeneration is relatively simple and commonly used in tissue culture protocols. Further, only the initial step in the culture protocol requires the addition of plant growth regulators (1 $mg.l^{-1}$ 2,4-D and 1 $mg.l^{-1}$ BAP) to the nutrient medium. In this way, the culture procedure is not as labour-intensive for this species as for many other bulbous species (Table 2.4). These findings are in contrast to previous studies on bulbous plants where bulblets were not obtained unless specific nutrient media were formulated (Bapat and Narayanaswamy, 1976; Jha *et al.*, 1984; Peck and Cumming, 1986).

High yields of *B. volubilis* plantlets can be obtained using the developed protocol and that these plantlets can be used to supplement the natural stock of plants in wild populations. Additionally, this study shows that a ready supply to the herbal medicinal trade is possible, which would alleviate pressure on existing resources already over-exploited by uncontrolled harvesting.

Chapter 3: ESTABLISHMENT OF A MICROPROPAGATION PROTOCOL FOR *Haworthia limifolia*, AND ELIMINATION OF VITRIFICATION IN REGENERATED *Haworthia limifolia* PLANTLETS

3.1. INTRODUCTION

3.1.1. The botany of *Haworthia limifolia*

The genus *Haworthia* belongs to family Liliaceae and, according to Court (1981), is closely related to the following genera: *Aloe*, *Chortolirion*, *Astroloba*, *Poellnitzia*, *Chamaealoe* and *Gasteria*. There have been many discrepancies as to how many South African *Haworthia* species there are, the most recent estimate being 67 (Court, 1981). Court (1981) describes *H. limifolia* as a large, well tubercled form. The ground colour of the fleshy leaves varies from dark-green in sheltered positions to russet-red shades in dry, exposed conditions. The outer leaf surface is covered with conspicuous pearly-white tubercles which are scattered or arranged in irregular transverse lines over the surface of the leaves (Plate 3.1). The flowers are whitish, narrowly tubular (<33 mm long and <10 mm across) and are carried in clusters on long, thin inflorescence stems.

3.1.2. The uses of *H. limifolia* in South Africa

According to Hutchings (1989), *Haworthia limifolia* is used in traditional medicines for both medicinal and magical purposes. Medicinal applications include the treatment of stomach ache, constipation, diarrhoea, worms, dysentery, nausea, indigestion and

haemorrhoids. Magical uses include placation of evil spirits, protection against enemies and storms, and to bring good luck to the user.

3.1.3. Modes of propagation

Haworthias may be propagated vegetatively from offsets from the parent plant or from leaf cuttings (Huxley, 1979; Sheat, 1987; Pienaar, 1992). Plants can also be propagated from fresh seed (Huxley, 1979; Berger, pers. comm.; Buckas pers. comm.) in a well-drained potting mixture. To date, there have been no published reports on the development of a protocol for the *in vitro* regeneration of *H. limifolia,* although several papers describing the establishment of micropropagation protocols for other *Haworthia* species have appeared in the literature (Table 3.1).

Table 3.1: Some of the reported studies of the development of *in vitro* regeneration protocols for *Haworthia* species

Species	Reference
H. arachnoides	Konishi *et al.* (1982)
H. comptonia	Rogers (1993a)
H. cymbiformis	Konishi *et al.* (1982)
H. emelyae	Rogers (1993b)
H. fasciata Haw.	Beyl and Sharma (1983)
H. mirabilis	Kemp and Stoltz (1981); Rogers (1993b)
H. mutica	Rogers (1993b)
H. planifolia var. cf. var. *setulifera* v. Poelln.	Wessels *et al.* (1976)
H. turgida	Majumdar (1970)
H. variegata	Majumdar and Schlosser (1972)

Plate 3.1: A mature *Haworthia limifolia* plant. Note the thick fleshy leaves and white tubercles arranged over the surfaces of the leaves. (bar = 12.5 mm)

3.1.4. Aims of the investigation

Haworthia limifolia is an important species commonly used in traditional medicine in KwaZulu/Natal and as a result, the harvestable size of this species has become smaller because of increasing demands (Twine, pers. comm.). Furthermore, wild populations of *H. limifolia*, as with most heavily sought after species, are declining. Preliminary investigations in this laboratory (Makwarela, pers. comm.) and by other workers (Mycock, pers. comm.) have resulted in a working protocol for the micropropagation of this species. The work in this study aimed to test this protocol and, in particular, to address the problem of vitrification previously encountered in regenerated plantlets.

3.2. MATERIALS AND METHODS

3.2.1. The production of aseptic explants

3.2.1.1. Preliminary assessment of endogenous contaminants

Leaves (50 mm x 20 mm) were excised from plants growing in pots and washed and surface sterilised. Sterilisation was carried out as described in Section 2.2.1.

3.2.1.2. Bacterial contamination

a) Growth of bacterial cultures

Thirty millilitres of sterile Luria-Bertani medium (10 $g.l^{-1}$ bactotryptone, 5 $g.l^{-1}$ yeast extract, 0.5 $g.l^{-1}$ NaCl, 2 $g.l^{-1}$ glucose) were inoculated with the bacterial strain/s detected in the preliminary investigation of contamination, using a flamed loop. Incubation was carried out for 24 hours at 28 °C and bacterial growth was detected by the optical turbidity of the medium.

b) Antibiotic testing

Several antibiotics and mixtures of antibiotics were tested (Table 3.2) in order to determine which had the highest efficacy against the bacterial strains present in the preliminary investigation (Table 3.2). The antibiotics tested were selected according to their mode of action on bacterial cells and whether they were specific for gram-negative or gram-positive strains. The efficacy of a particular antibiotic was determined by measuring the diameter of the zone of inhibition (Figure 3.1, Table 3.2) obtained when the bacterial culture was spread on the surface of a nutrient medium (Luria-Bertani medium solidified with 10$g.l^{-1}$ agar) in a Petri dish (48 hours at 28 °C).

The zone of inhibition is described as the clear area around the antibiotic well denoting inhibition of bacterial cell growth (Figure 3.1).

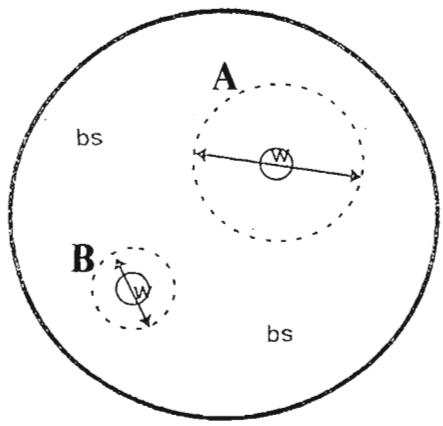


Figure 3.1: Diagrammatic representation of the zone of inhibition (⟷) obtained during antibiotic screening tests. **A**: more efficient antibiotic with a larger zone of inhibition than **B**, a less efficient antibiotic; **w**: well containing antibiotic of known concentration; **bs**: bacterial spread.

3.2.1.3. Testing of anti-fungal agents

Benlate (benomyl; Effecto SA) (0.1 $g.l^{-1}$) and Previcur N (probamacarb hydrochloride; FBC Holdings) (0.36 $g.l^{-1}$) were added, either separately or in combination, to MS nutrient medium supplemented with 20 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. An overnight wash solution (MS nutrients and 20 $g.l^{-1}$ sucrose) containing either, or a combination, of the two fungicides, was also tested for efficacy against endogenous fungal contaminants. Leaf explants (4 - 5 mm^2) were cultured onto MS nutrient medium supplemented with 20 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar, after overnight washing.

3.2.1.4. Final sterilisation protocol

Leaf material, obtained from plants grown in pots, was surface-sterilised for 2 - 3 minutes in 70% [v/v] ethanol before being washed in a solution of 1% [v/v] NaOCl for 20 minutes. The leaves were then rinsed three times with sterile water after which explants (4 - 5 mm^2) were cultured onto nutrient medium containing 20 $g.l^{-1}$ sucrose, 0.1 $g.l^{-1}$ Benlate, 50 $mg.l^{-1}$ Rifampicin, 25 $mg.l^{-1}$ Trimethoprim and 10 $g.l^{-1}$ agar.

3.2.2. Somatic embryogenesis of *H. limifolia*

3.2.2.1. Induction of embryo formation

Leaf explants approximately $5mm^2$, were cut from sterilised leaves (Section 3.2.1.4) and placed on induction medium in 65 mm Petri dishes (3 explants per dish). The medium consisted of MS macro- and micro-nutrients, BS (Beyl and Sharma, 1983) vitamins and amino acids, 20 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. At this stage, the basal medium was supplemented with 50 $mg.l^{-1}$ Rifampicin, 25 $mg.l^{-1}$ Trimethoprim, 0.1 $g.l^{-1}$ Benlate and 1 - 5 $mg.l^{-1}$ 2,4-D or 1 - 3 $mg.l^{-1}$ picloram and 0.25 $mg.l^{-1}$ kinetin. Cultures were incubated in the dark at 25 °C for 14 weeks, after which time explants were examined for the presence of embryonic cells and structures. The percentage of explants producing embryos was recorded.

3.2.2.2. Embryo maturation and plantlet germination

Explants that developed embryos were subcultured and transferred to the basal medium devoid of growth regulators and antibiotics. The cultures were maintained under a 16:8 h photoperiod (200 $\mu Em^{-2}s^{-1}$) at 25 °C. Regenerated plantlets were excised from the original explant and cultured onto the basal medium devoid of

growth regulators for growth (4 - 5 weeks), whereafter they were hardened-off as described below (Section 3.2.3).

Table 3.2: Antibiotics tested for bacteriostatic efficacy against the endogenous bacterial contaminants observed in the preliminary investigation.

Antibiotic/s	Concentration ($\mu g.ml^{-1}$)	Effective against Gram +ve or -ve
Gentamycin	50	-ve
	100	
Streptomycin	50	+ve
	100	
Ampicillin	50	+ve and -ve
	100	
	200	
Rifampicin	50	+ve and -ve
	100	
Kanamycin	50	+ve and -ve
	100	
Trimethoprim	25	+ve
	50	
PenStrep	50	+ve and -ve
	100	
	200	
Rifampicin : Trimethoprim	25 : 25	+ve and -ve
	50 : 25	
Streptomycin : Gentamycin	50 : 50	+ve and -ve
	100 : 100	
Streptomycin : Rifampicin	100 : 50	+ve and -ve
Kanamycin : Rifampicin	50 : 50	+ve and -ve
Ampicillin : Rifampicin	50 : 50	+ve and -ve
Streptomycin : Kanamycin	100 : 50	+ve and -ve
Kanamycin : Ampicillin	50 : 50	+ve and -ve
PenStrep : Tetracycline	50 : 25	+ve and -ve

3.2.3. Hardening-off of regenerated plantlets

Plantlets were transferred to sterile potting soil and maintained under different conditions, as listed below, to test the most efficient hardening-off protocol. Within each treatment, the humidity of the microclimate was decreased gradually until the plantlets were acclimatised after 3 - 4 weeks. The treatments used to test for optimum *ex vitro* survival of plantlets were:

1) Plantlets placed in sterile culture bottles (65 mm × 70 mm) containing potting soil and the culture vessel was sealed from the external environment with a plastic lid; or

2) plantlets were placed in sterile culture bottles (65 mm × 70 mm) containing potting soil and culture vessels were sealed with transparent plastic pierced with holes to allow gaseous exchange (surviving plantlets were transferred to seedling trays after 2 weeks); or

3) plantlets were placed in seedling trays containing moist soil, mist- sprayed daily with tap water and covered with plastic; or

4) plantlets were placed in seedling trays containing dry soil, mist- sprayed daily with tap water and covered with plastic; or

5) plantlets were placed in seedling trays containing dry soil and plantlets were mist-sprayed daily with tap water.

3.2.4. Vitrification studies

3.2.4.1. Changes in medium composition and physical environment on vitrification

Because of substantial losses of plantlets during hardening-off as a result of vitrification, several treatments were tested to reverse this condition. The treatments

included changes in medium composition and culture environment (Table 3.3). Single plantlets were placed onto medium in culture tubes (20 mm x 100 mm) or culture bottles (65 mm x 70 mm), depending on the treatment, and after 3 - 4 weeks, both visual and microscopic examinations were used to determine which treatment reduced or reversed the vitrified condition.

Table 3.3: Constituents added to the culture medium to reverse plantlet vitrification. Plantlets were placed onto nutrient medium (MS macro- and micro-nutrients, BS vitamins and amino acids, 20 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar) and incubated in the light (16:8 h photoperiod, 200 $\mu Em^{-2}s^{-1}$). n (number of plantlets) = 15 per treatment.

Constituent	Concentration ($g.l^{-1}$)
Gelrite	4.0
	8.0
Agar	12.0
$CoCl_2$	0.00025
	0.0005
NH_4^+	1.25
	0
K^+	0.0016
Ca^{2+}	0.88
	1.22
Phloroglucinol	0.060
	0.12

In addition to the additives listed in Table 3.3, plantlets were also incubated (Plate 3.2) with containers of silica gel in culture bottles (65 mm x 70 mm). This treatment served to reduce the humidity within the culture vessel.

Plate 3.2: Incubation of plantlets with container of silica gel to reduce relative humidity within the culture vessel. (bar = 7.5 mm)

3.2.4.2. Wax embedding and sectioning protocols for anatomy studies

After 2 - 3 weeks, 1 - 2 mm thick transverse sections of the leaves from vitrified and normal plantlets from all treatments were cut and embedded in wax according to the following protocol:

1. Material was fixed in FAA overnight (FAA: 50:5:10:35; ethanol (95% [v/v]), glacial acetic acid, formaldehyde (40% [v/v]), distilled water);
2. This was followed by immersion in a series of ethanol (50% [v/v], 70% [v/v], 90% [v/v]) for 1 hour each, followed by 100% [v/v] ethanol with 3 changes (1 hour each) and one change overnight;
3. Material was then immersed in a series of ethanol (100% [v/v] and xylene (100% [v/v]): (75:25, 50:50, 20:80) for 1 hour each, followed by 100% [v/v] xylene with 3 changes(1 hour each) and one change overnight;
4. Initial infiltration was achieved by adding wax chips one or two at a time at room temperature until the xylene:wax mixture was saturated;
5. Material was further infiltrated by placing on a hotplate at a warm temperature with the addition of more wax;
6. The preparations were placed in an oven at 60 °C for 1 hour;
7. The material was placed in pure molten wax and then placed in oven at 60 °C for 1 hour;
8. Wax was replaced twice more (infiltration in oven for 1 hour each time);
9. Sections were transferred to the base of a plastic ice-cube tray and hot molten wax was poured over to produce wax blocks;
10. Blocks were cooled and solidified overnight at room temperature.

The wax blocks obtained using the above protocol, were sectioned using an 820 Rotary Microtome (sections were 3 - 5 μm thick). The sections, several of which were placed on each microscope slide, were dewaxed according to the procedure listed below:

Each slide was washed in the following solutions for 5 minutes:

1. Two xylene (100% [v/v]) rinses;
2. 100% [v/v] xylene:100% [v/v] ethanol;
3. A series of ethanol (95% [v/v], 75% [v/v], 65% [v/v], 50% [v/v], 30% [v/v]);
4. Distilled water.

The slides were allowed to dry after the dewaxing procedure, after which they were stained with 1% [m/v] lactophenol blue for 20 minutes. The excess stain was washed off by dipping the slides into a beaker of distilled water. They were then dried thoroughly before microscopical viewing.

3.2.5. Microscopy and Photography

The embryos produced were examined microscopically using a Wild M3 stereo-microscope and a Nikon Biophot light microscope, and the stages of embryo development and germination were photographically recorded using a Wild photoautomat MPS 55 system and a Nikon FX 35A and UFX photorecording system. Gross morphological characteristics of the embryos and plantlets were also recorded photographically using a Nikon FM2 camera with a Mikro Nikkor 60 mm macro lens.

Leaf anatomy was recorded using the Nikon FX 35A and UFX photorecording system.

3.2.6. Analysis of data

The extent of embryo formation, plantlet production and vitrification was recorded and average values obtained. Experiments were repeated at least three times with the number of explants varying between 15 and 50 depending on the experiment being carried out. Where appropriate, differences in the mean percentages of embryo formation, percentages of explants which produced plantlets, or percentage of vitrified plantlets, were assessed using one-way ANOVA (Statgraphics Plus, 1993). Alphabetical values were assigned to the means obtained for each treatment, different letters indicating significant differences.

3.3. RESULTS

3.3.1. Production of sterile explants

3.3.1.1. Initial tests for contamination

Explants were cultured onto MS nutrient medium as described in Section 3.2.1. The standard sterilisation treatment of the explants (20 minutes in 1% [v/v] NaOCl and Tween-20 followed by three sterile washes with water) was ineffective in eliminating bacterial contamination. Visible growth of micro-organisms was evident five days after culture initiation.

3.3.1.2. Antibiotic screening tests

Antibiotic screening tests were performed using them individually, or as mixtures (Table 3.4). A combination of Rifampicin (50 $\mu g.ml^{-1}$) and Trimethoprim (25 $\mu g.ml^{-1}$) was found to have the greatest bacteriostatic activity on the contaminants isolated from the initial leaf cultures. Rifampicin in combination with Streptomycin, Kanamycin and Ampicillin resulted in a decreased bacteriostatic effect relative to when in combination with Trimethoprim (Table 3.4). Ampicillin did not inhibit bacterial cell growth to any extent at the concentrations tested. As a result of the sensitivity test findings, a combination of Rifampicin (50 $\mu g.ml^{-1}$) and Trimethoprim (25 $\mu g.ml^{-1}$) was incorporated into the culture medium and was effective in decreasing bacterial contamination significantly.

Table 3.4: The effect of different antibiotics and combinations of antibiotics on inhibition of bacterial growth isolated from initial cultures of *H. limifolia* plantlets. The diameter of the zone of inhibition was measured 48 hours after the onset of incubation at 28°C.

Antibiotic/s	Concentration ($\mu g.ml^{-1}$)	Diameter of zone of inhibition (mm)
Gentamycin	50	18
	100	24
Streptomycin	50	25
	100	25
Ampicillin	50	0
	100	10
	200	10
Rifampicin	50	22
	100	24
Kanamycin	50	17
	100	19
Trimethoprim	25	36
	50	36
PenStrep	50	23
	100	24
	200	23
Rifampicin : Trimethoprim	25 : 25	35
	50 : 25	38
Streptomycin : Gentamycin	50 : 50	22
	100 : 100	23
Streptomycin : Rifampicin	100 : 50	22
Kanamycin : Rifampicin	50 : 50	21
Ampicillin : Rifampicin	50 : 50	21
Streptomycin : Kanamycin	100 : 50	22
Kanamycin : Ampicillin	50 : 50	20
PenStrep : Tetracycline	50 : 25	20

3.3.1.3. Assessment of the efficacy of antifungal agents

Suppression of bacterial growth by the addition of antibiotics, however, allowed the proliferation of fungal contaminants. Similar findings have been reported by other workers (for example Falkiner, 1990) who reported that fungal growth was stimulated by prevention of bacterial growth. Consequently, antifungal agents were tested (Table 3.5) for their efficiency in reducing or eliminating this problem. Of the two fungicides tested, Benlate (0.1 $g.l^{-1}$) in the culture medium resulted in a significant reduction of fungal contaminants (by 92.5%), whereas Previcur N (0.36 $g.l^{-1}$) reduced contamination to a lesser extent (by 42.3 %). The two fungicides used in combination appeared to act antagonistically resulting in enhanced fungal growth compared with Benlate or Previcur N alone. Using the fungicides in a 24 h wash solution did not inhibit fugal growth to the same degree as when they were used only in the culture medium (Table 3.5).

Table 3.5: The effect of Benlate (0.1 $g.l^{-1}$) and Previcur N (0.36 $g.l^{-1}$) on the reduction of fungal growth in leaf cultures of *H. limifolia*. Explant material was either cultured directly onto medium containing fungicide/s or was washed before being cultured onto the same medium. n= 40 - 45.

Fungicide	24 hour fungicide wash (Y/N)	% fungal contamination
none	-	70.4^{f}
Benlate	N	5.3^{a}
Previcur N	N	40.6^{c}
Benlate and Previcur N	N	58.7^{d}
Benlate	Y	37.9^{b}
Previcur N	Y	76.2^{g}
Benlate and Previcur N	Y	65.7^{e}

3.3.2. Production of plantlets via somatic embryogenesis

3.3.2.1. Somatic embryogenesis

A very small amount of clear, almost crystalline callus was formed on the cut surface of explants after four to five weeks in culture (Plate 3.3A). Embryogenic structures originated *in vitro* almost without an intervening callus phase indicating direct somatic embryogenesis. After 5 - 6 weeks, extremely dense, yellow dome-shaped embryoids appeared (Plate 3.3B). Large clusters of embryogenic cells, globular-, heart- and torpedo-shaped embryos (Plate 3.3C) were observed on the explants. Plantlet germination was not observed upon transfer to induction medium devoid of growth hormones. Rather, callus proliferation occurred (secondary embryogenesis - Plate 3.3D) followed by the production of secondary embryos after 6 weeks which germinated (Plate 3.3E) to produce plantlets (Plate 3.3F) 14 weeks after the onset of culture initiation i.e. secondary embryogenesis was indirect. Plantlets were dissected out (Plate 3.3G) from the callus and transferred to basal medium without growth regulators for growth (4 - 5 weeks) as described in Section 3.2.3.2. Healthy plantlets were transferred to seedling trays containing potting soil to promote hardening-off and growth (Plate 3.3H).

3.3.2.2. The effect of growth regulators on embryo formation

In order to determine the optimum concentration of auxin (2,4-D and picloram) promoting embryo formation on leaf cultures of *H. limifolia*, several induction media were tested (Table 3.6). The results indicated that increased 2,4-D concentrations in the induction medium resulted in an increase in the percentage of explants producing embryos. The highest percentage of explants producing embryos (73.3%) after 12

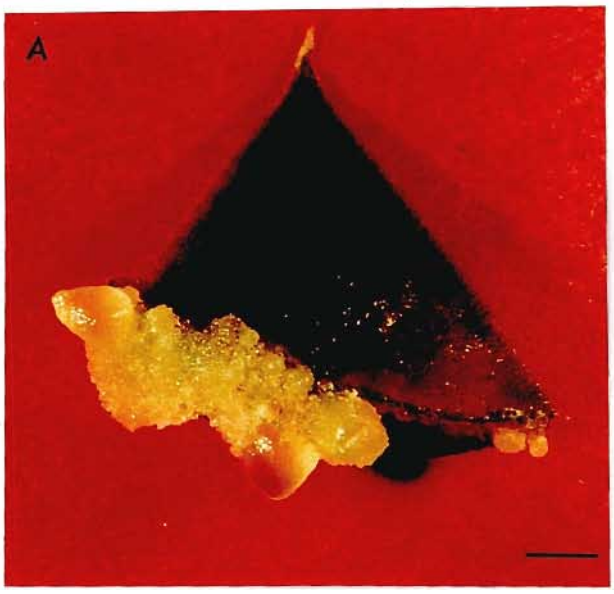
weeks in culture, was observed when explants were placed on induction media supplemented with 3 $mg.l^{-1}$ 2,4-D (Table 3.6). A significantly lower percentage of explants produced embryos when the medium was supplemented with picloram (1 $mg.l^{-1}$ and 3 $mg.l^{-1}$) alone or with picloram in combination with 0.25 $mg.l^{-1}$ kinetin.

Table 3.6: Effect of growth regulators on the induction of somatic embryogenesis on leaf explants of *H. limifolia* after 6 and 12 weeks in culture. Induction media contained MS nutrients, 20 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. n=42 - 45 explants/treatment.

Growth Regulator ($mg.l^{-1}$)	% explants with callus	
	6 weeks	12 weeks
2,4-D		
1	42.8^{k}	61.9^{l}
3	40.0^{j}	73.3^{n}
5	33.3^{i}	63.3^{m}
picloram		
1	10.2^{a}	15.4^{b}
3	27.3^{e}	28.1^{f}
picloram and 0.25 $mg.l^{-1}$ kinetin		
1	18.3^{c}	23.5^{d}
3	30.7^{g}	32.3^{h}

Plate 3.3: Stages of somatic embryogenesis in *Haworthia limifolia*

(A) A small amount of clear, crystalline callus derived from leaf explant *of H. limifolia*. Note the already-formed dense embryos (e) embedded in the tissue. Explants were cultured on induction medium containing MS macro- and micro-nutrients and BS vitamins and amino acids, 20 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar and 3 $mg.l^{-1}$ 2,4-D. Bar = 2 mm. (B) Easily distinguished cluster of primary embryos. Bar = 2 mm. (C) Globular-, heart- and torpedo-shaped primary embryos produced directly from the mesophyll tissue. Bar = 2 mm. (D) Callus proliferation and secondary embryo production and germination on basal medium. Bar = 20 mm. (E) Mature shoot (s) and root (r) primordia of a secondary embryo. Bar = 2 mm. (F) Germinating plantlet on secondary embryo callus medium. Bar = 2 mm. (G) Cluster of plantlets dissected out from secondary embryos callus stage. Bar = 3 mm. (H) Hardened-off plantlets acclimatised to greenhouse conditions. Bar = 20 mm.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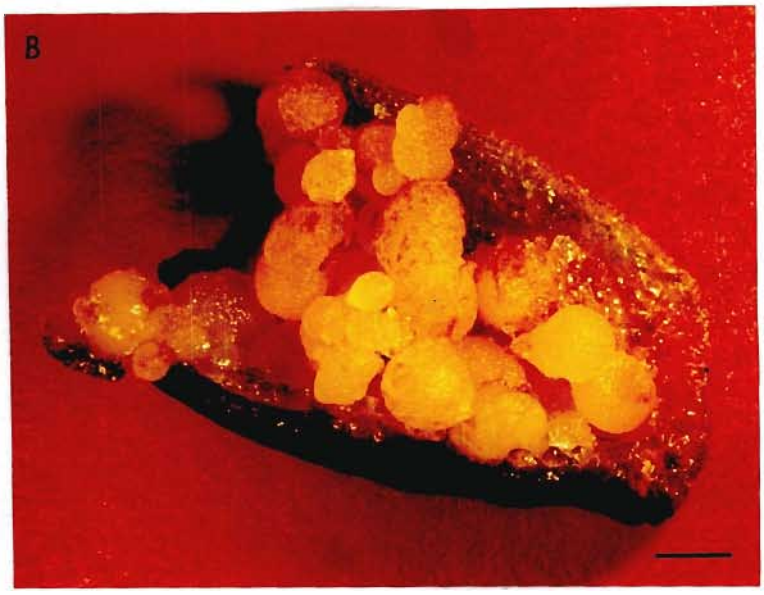
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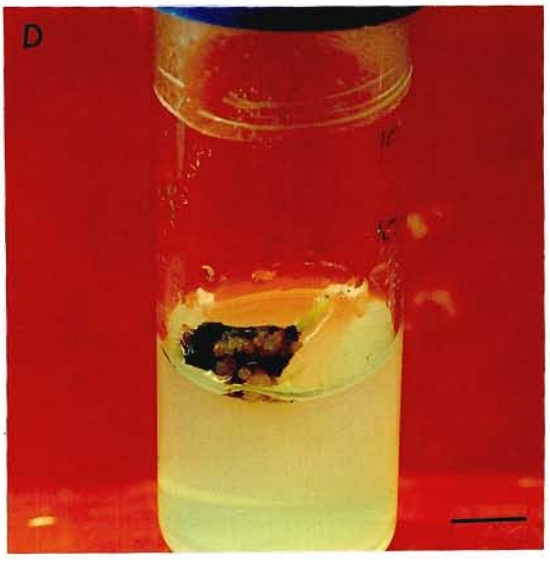
C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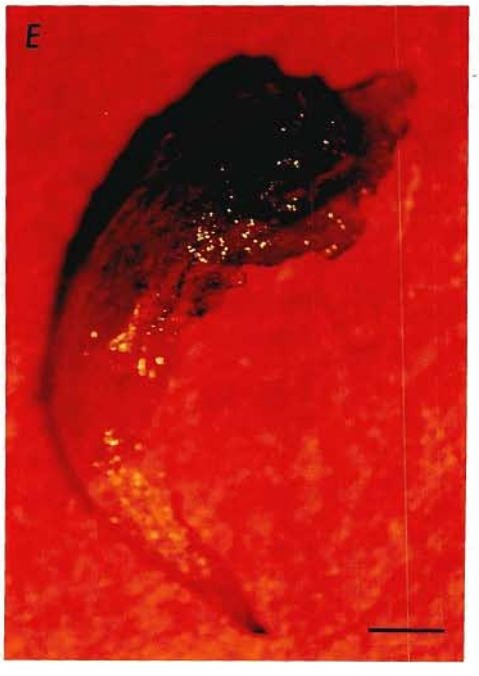
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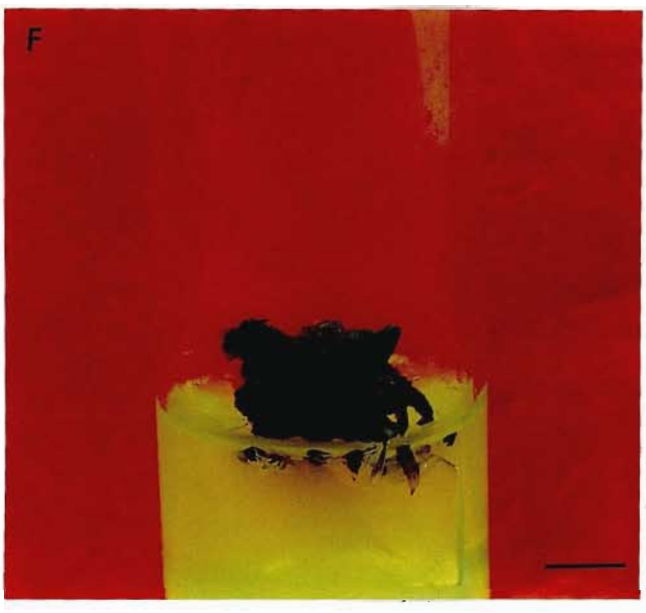

F


G


H

3.3.2.3. Plantlet regeneration and hardening-off

As described in Section 3.2.2.2, subsequent to somatic embryogenesis, explants with embryos were transferred to nutrient medium devoid of growth regulators. Plantlet regeneration from secondary embryos, occurred 10 - 12 weeks after transfer to this medium. More than 20 plantlets were produced from each explant producing callus. Healthy plantlets produced *in vitro* were transferred to either sterile bottles containing soil or directly into seedling trays containing soil as described in Section 3.2.4. The results obtained (Table 3.7) indicated that the most appropriate hardening-off procedure giving the highest plantlet survival rate (85.4%), was the planting-out of plantlets directly into slightly moist soil in seedling trays or small pots. A twice-daily mist-spray was required to maintain humidity and prevent plantlets from drying out completely. Plants in dry soil had a slightly lower survival rate (70.6%). In contrast, only 9.7% of plantlets planted out in soil in culture bottles survived. The rest of the plantlets rotted, apparently due to excess moisture.

Table 3.7: Survival of *in vitro* regenerated *H. limifolia* plantlets under various hardening-off treatments

n (number of plantlets) = 32 - 35 per treatment. All plantlets were planted out in potting soil for each treatment.

Treatment	% survival (4 weeks)
Sterile glass bottles (plastic lids)*	9.7^{a}
Sterile glass bottles (aerated lids)*	10.2^{b}
Seedling trays (moist conditions-covered)	50.3^{c}
Seedling trays (dry conditions-covered)	62.3^{d}
Seedling trays (moist conditions + mist-spray-no cover)	85.4^{f}
Seedling trays (dry conditions + mist-spray)-no cover	70.6^{e}

*: Plantlets planted into sterile potting soil and survivors subsequently transferred into seedling trays.

As mentioned in Section 3.2.4, a major problem encountered in micropropagation of *H. limifolia*, was the large proportion (60%) of vitrified (or hyperhydric) plantlets regenerated. Whereas, healthy plantlets were identified as being opaque and dark-green in colour, vitrified plantlets appeared glassy and in extreme cases were almost totally translucent (Plate 3.4).

Plate 3.4: The morphology of vitrified and normal *Haworthia limifolia* plantlets Note the glassy, almost translucent appearance of leaves of the vitrified plantlet compared with the dark-green, opaque appearance of the normal plantlet. Bar = 7.5 mm.

Vitrified plantlets could not be hardened-off using any of the protocols tested in Section 3.2.4. One hundred percent mortality was observed one week after planting out. In comparison, 85.4% of healthy plantlets could be hardened-off and acclimated to ambient conditions using the appropriate protocol.

3.3.3. Effect of changes in culture environment on the reversal of hyperhydricity

Hyperhydricity, or vitrification, results from metabolic perturbations (for example hypolignification, hyperhydricity) induced by the combined action of several physical (for example gel solidity and temperature) and chemical factors (for example ammonium and cytokinins) of the culture environment (DeBergh, 1983; DeBergh *et al.*, 1992; Paques and Boxus, 1987; Ziv, 1991). Several workers have established that physiological vitrification can be reversed by manipulating the culture medium and/or environment (DeBergh *et al.*, 1981; DeBergh, 1983; Bornmann and Vogelmann, 1984; Gaspar and Kevers, 1985; Paques *et al.*, 1985) and several culture conditions. The nature and concentration of gelling agent and of $CoCl_2$, K^+, Ca^{2+} and NH_4^+ were manipulated. Also phloroglucinol was added to the medium and silica gel was introduced into the culture vessel, in attempts to induce a reversal of the observable vitrification characteristics. Light microscopy was used to ascertain whether the treatments applied had any positive effect on the restoration of cellular structure and tissue organisation.

Plantlets were identified as being either healthy or vitrified. Healthy plantlets (Figure 3.2A) were opaque and dark green with the white tubercles, characteristic of this species (Plate 3.1), present on the surface of the leaves. In contrast, vitrified plantlets

(Figure 3.2B) appeared translucent or "glassy", and pale green or almost colourless, in extreme cases.

The anatomical characteristics exhibited by the leaves of vitrified plantlets were the presence of large intercellular air-spaces, the absence of the vascular system, reduced numbers of palisade cell layers, lack of cuticle and an abnormal, often discontinuous epidermal layer (Figure 3.2G and 3.2H). These features can be contrasted with those of healthy plantlets (Figure 3.2C and 3.2D). In addition, vitrified leaves also showed a pronounced lack of chloroplasts compared with those of normal plants. Of the treatments tested, the use of Gelrite (6 $g.l^{-1}$ and 8 $g.l^{-1}$) as a solidifying medium, an increased K^+, $CoCl_2$ and Ca^{2+} concentration and a decrease in the level of NH_4^+ in the culture medium as recommended by Beauchesne, 1981, DeBergh *et al.*, 1992, Letouzé and Daguin, 1983 and Ziv *et al.*, 1987, did not have any effect on reducing or reversing the abnormal characteristics brought about by the vitrification process. Furthermore, phloroglucinol (as recommended by Hedegus and Phan, 1983), when added to the culture medium, not only maintained the vitrified appearance of plantlets, but also was associated with plant death within 2 - 3 weeks. Vitrified plantlets cultured onto media solidified with 12 $g.l^{-1}$ agar (instead of 10 $g.l^{-1}$), appeared to become more green and were less translucent than vitrified plantlets after 2 - 3 weeks. Microscopic examination of the leaves (Figure 3.2E and 3.2F) revealed that the cuticle had an abnormal appearance (wavy edge) compared with normal plantlets. Both the palisade and mesophyll layers appeared normal. Plantlets cultured in the presence of silica gel, which reduced humidity within the culture vessel, had normal anatomical

characteristics (Figure 3.2C and 3.2D). These plantlets could be easily hardened-off according to the method described in Section 3.2.4.

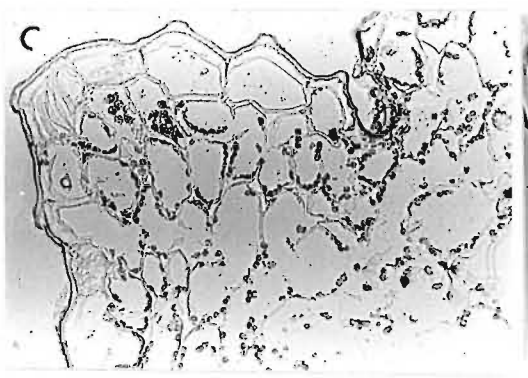
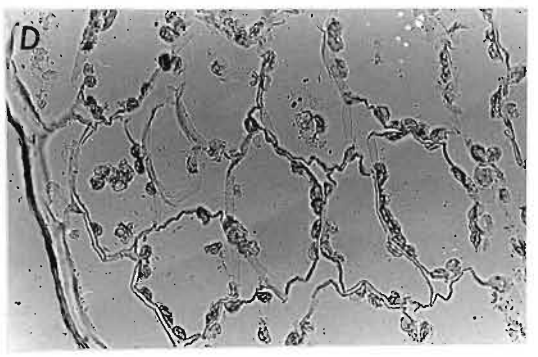
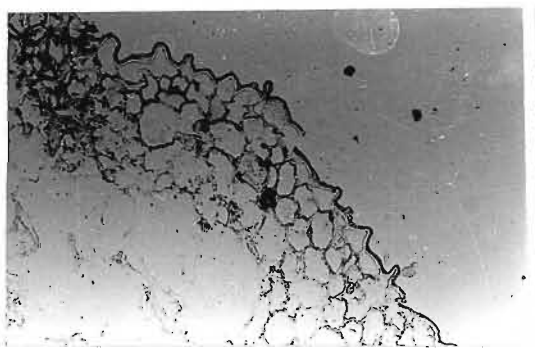
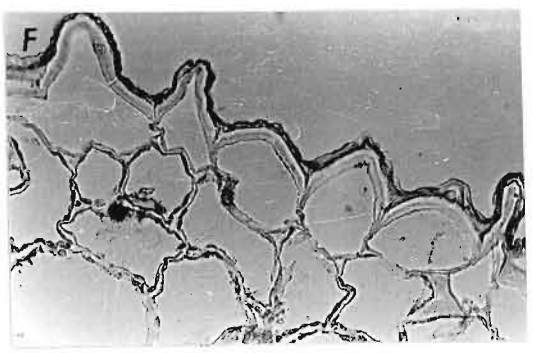
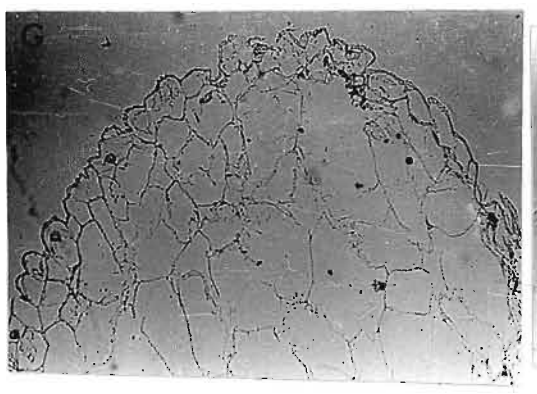
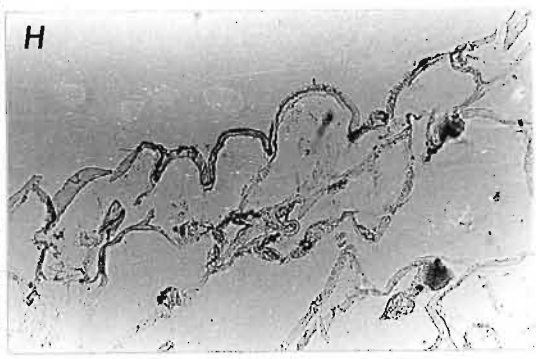
Treatment	Gross morphology	Leaf Anatomy
silica gel placed on top of the agar		 
agar (12 g.l^{-1}) only		 
agar (10 g.l^{-1}) only Gelrite (4 and 8 g.l^{-1}) $CoCl_2$ (0.00025 and 0.00050 g.l^{-1}) NH_4^+ (1.25 and 0 g.l^{-1}) K^+ (0.00166 g.l^{-1}) Ca_2^+ (0.88 and 1.22 g.l^{-1}) phloroglucinol (0.060 and 0.12 g.l^{-1})		 

Figure 3.2: Anatomical and morphological characteristics of vitrified *Haworthia limifolia* plantlets after various treatments tested to reduce or reverse vitrification in *in vitro* regenerated plantlets. Plantlets were incubated on nutrient medium containing MS macro- and micro-nutrients, B5 vitamins and amino acids, 20 g.l^{-1} sucrose and cultured in the light (16:8 hour photoperiod; 200 Em^{-2}s^{-1}). A: Normal, healthy plantlet cultured in the presence of silica gel or medium solidified with 12 g.l^{-1} agar (bar = 15 mm); B: Vitrified plantlet cultured in the presence of agar (10 g.l^{-1}), Gelrite (4 and 8 g.l^{-1}), $CoCl_2$ (0.00025 and 0.00050 g.l^{-1}), NH_4^+ (1.25 and 0 g.l^{-1}), K^+ (0.00166 g.l^{-1}), Ca_2^+ (0.88 and 1.22 g.l^{-1}) and phloroglucinol (0.060 and 0.12 g.l^{-1}) (bar = 15 mm); C and D: Anatomy of plantlets cultured in the presence of silica gel (C: 570x; D: 1140x); E and F: Anatomy of plantlets after culture on medium solidified with 12 g.l-1 agar (E: 285x; F: 1140x), G and H: Anatomy of plantlets cultured on agar (10 g.l^{-1}), Gelrite (4 and 8 g.l^{-1}), $CoCl_2$ (0.00025 and 0.00050 g.l^{-1}), NH_4^+ (1.25 and 0 g.l^{-1}), K^+ (0.00166 g.l^{-1}), Ca_2^+ (0.88 and 1.22 g.l^{-1}) and phloroglucinol (0.060 and 0.12 g.l^{-1}) (G: 285x; H: 1140x)]

3.4. DISCUSSION

3.4.1. Micropropagation of *Haworthia limifolia*

The protocol tested in this study (Figure 3.3) produced extremely high yields of plantlets - more than 20 plants were regenerated from each explant producing secondary embryos.

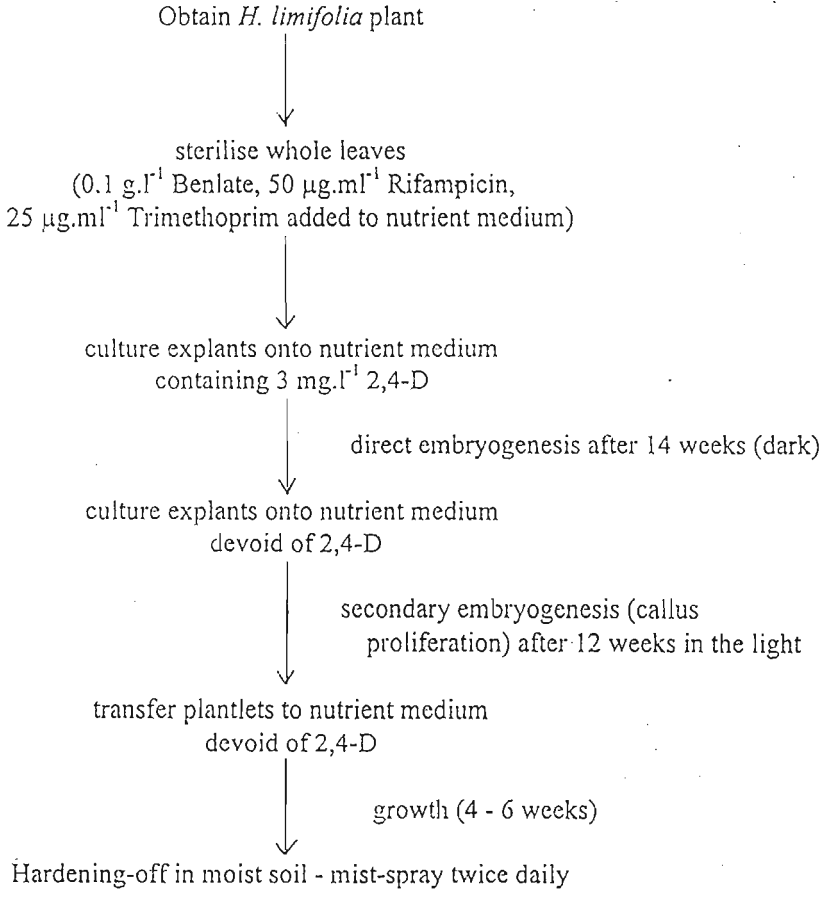


Figure 3.3: Schematic representation of somatic embryogenesis in *Haworthia limifolia*

The results of this study confirmed those that were undertaken previously in our laboratory (Makwarela pers. comm.). In *H. limifolia*, embryogenesis proceeded directly from somatic cells of the cultured explants, with the embryos originating *in vitro* without an intervening callus phase (Plate 3.3A and 3.3B). However, removal of 2,4-D from the culture medium did not induce embryo maturation and germination and subsequent plantlet regeneration. Instead, extensive callus proliferation occurred from which secondary embryos developed and germinated into plantlets (Plate 3.3D and 3.3G). This process is termed secondary embryogenesis and has been reported for other *Haworthia* species (Beyl and Sharma, 1983), but the explanation for the production of secondary embryos is unknown. The studies conducted by Beyl and Sharma (1983) indicated that 2,4-D used at any concentration, produced low yields of embryos compared with a combination of picloram and kinetin. In contrast, in this study, a larger percentage of explants cultured on 2,4-D produced embryos than explants cultured on picloram and kinetin (Table 3.6). Somatic embryogenesis, in many species, can be enhanced by the addition of activated charcoal to the maturation medium (Fridborg and Eriksson, 1975; Drew, 1979) as it adsorbs substantial amounts of auxins, high levels of which prevent embryo germination. Similarly, 5-hydroxymethylfurfural, an inhibitor which is formed by the degradation of sucrose during autoclaving, is also adsorbed. Fridborg *et al.* (1978) have also found that media containing activated charcoal had a lower level of phenylacetic and p-hydroxy benzoic acids which inhibit somatic embryogenesis when present in cultures. In previous studies (Makwarela pers. comm.) on *Haworthia limifolia*, activated charcoal did not improve somatic embryogenesis so this approach was not used in the present study.

3.4.2. Vitrification studies

The terms, vitrification or hyperhydricity, are used to describe organs and tissues having abnormal morphological and anatomical characteristics and physiological function. In this discussion, the two terms are used interchangeably. The morphological (glassy, translucent leaf appearance) and anatomical (severe defects at the microscopic level) symptoms have been reviewed by DeBergh *et al.* (1992) and avoidance or reversal of these conditions is achieved by controlling the composition of the culture medium, environmental conditions and quality of the cultured explants. In this study, the changes in the medium composition that were tested included either an increase in the concentration of $CoCl_2$, K^+ or Ca^{2+}, or a decrease in the NH_4^+ concentration and the addition of phloroglucinol (Table 3.3). The relative humidity within the culture environment was altered by using Gelrite (instead of agar) as the solidifying medium, a higher concentration of agar (12 $g.l^{-1}$) than the usually-used concentration (10 $g.l^{-1}$) and culture of plantlets in the presence of silica gel (Table 3.3).

The changes to the culture medium did not reverse the vitrified condition in any of the plantlets tested and the leaves of the hyperhydric plantlets appeared glassy and translucent (Plate 3.4 and Figure 3.2B). Similar findings have been reported by DeBergh *et al.* (1981) and Aitken-Christie and Jones (1987). Those authors have also reported other morphological characteristics such as short internodes, rosetted leaves, changes in stem diameter and thick, elongated, wrinkled or curled leaves for *Pinus* spp., *Cynara scolymus, Forsythia intermedia, Oreopanax nympaelifolium,* and *Gerbera jamesonii.*

In this investigation, an evaluation of the leaf anatomy of *H. limifolia* plants cultured under these conditions (i.e. a change in medium composition) revealed that serious defects, as compared to healthy plantlets, included an absence of the cuticle layer, a reduced number of palisade cells, absence of chloroplasts and the presence of large intercellular air spaces in the mesophyll layer (Figure 3.2G and 3.2H). These characteristics have also been reported for vitrified *Salix babylonica* (Beauchesne, 1981; Letouzé and Daguin, 1983), plum (Brainerd and Fuchigami, 1981), *Dianthus caryophyllus* (Kevers and Gaspar, 1985; Ziv, 1991), *Malus* spp. (Paques and Boxus, 1987) and *Saintpaulia* (Ziv, 1991). In addition to these abnormalities, changes in the composition and structure of the surface wax and cuticle have also been reported in *Dianthus caryophyllus* by Earle and Langhans (1975) and Sutter and Langhans (1982) and Grout and Asten (1977) have also shown that the vascular connection between the roots and shoots is interrupted.

Apart from changes in the morphological and anatomical characteristics, biochemical pathways are also affected by the hyperhydric condition (Gaspar *et al.*, 1987; Ziv, 1991). A considerable amount of work has been done to attempt to evaluate the role played by different enzymes and mineral elements in the development of hyperhydricity and Gaspar *et al.* (1987) and Ziv (1991) have reported that vitrified shoots and leaves have less lignin and cellulose and the activity of various enzymes is lowered relative to normal tissues. None of this type of study was attempted in this investigation.

agent concentration is that propagation rates decrease markedly as a result of the restricted availability of nutrients to the culture material. Other additives which have been found to reverse hyperhydricity are the use of pectin (Zucherelli, 1979), phloridzin or phloroglucinol (Jones, 1976; Hedegus and Phan, 1983), Ni^{2+}, fructose or galactose as carbon source and methionine or $AgNO_3$ (DeBergh *et al.*, 1992). Of these, phloroglucinol was used resulting in plantlet death after 2 - 3 weeks.

Containers filled with silica gel (as shown in Plate 3.2) and placed in the culture vessel did cause a reversal of the morphological characteristics of the leaves of vitrified plantlets after 4 - 6 weeks in culture: the leaves became opaque and green and microscopical examination revealed that the plantlets subjected to this treatment had a normal leaf anatomy (Figure 3.2C and 3.2D) and were readily hardened-off. Similar findings for the reduction of humidity within culture vessels, have been reported by Wardle *et al.* (1983) for *Chrysanthemum morifolium* cultures. Those authors reduced the relative humidity within the culture vessel by placing a layer of lanolin over the surface of the agar or by suspending bags of silica gel within the culture vessel. Both of these treatments reduced relative humidity in *C. morifolium* cultures thereby reversing vitrification.

Other environmental factors such as temperature, light intensity and quality and air movement in the culture vessel can also influence plant growth in culture. Gradients of temperature, for example, have also been found to influence relative humidity within the culture container [Brainerd and Fuchigami, 1981 (*Malus* spp.); Short *et al.*, 1987 (*Brassica* spp.)]. Boxus *et al.* (1978) also placed *Prunus* and *Malus* cultures

without leaves in cold storage (2 - 4 °C) to prevent hyperhydricity. Other workers have cooled the shelves on which containers are placed to create a temperature gradient within the container and reduce water vapour in the head space (Vanderschaeghe and DeBergh, 1987). Increased light intensity has also been successful (DeBergh *et al.*, 1992).

3.4.3. Future research strategies and conservation of the species

Although large numbers of plantlets of *Haworthia limifolia* can be propagated via somatic embryogenesis, the length of time from the onset of culture initiation to the planting-out of regenerated plantlets is protracted (± 30 weeks). Similar findings have been reported by Ogihara (1981) and Beyl and Sharma (1983) for other *Haworthia* species. The development of a protocol for the direct plantlet regeneration from primary embryos would be of obvious benefit towards reducing the time in culture. Embryo maturation and subsequent plantlet regeneration from the embryos formed after 14 weeks would reduce the regeneration time by 50%. Ogihara (1981), found that extensive somaclonal variation in *Haworthia* plants regenerated from 2-year old callus occurred but, in comparison, Rogers (1993b) reported that plants regenerated via organogenesis directly from leaf explants, without passing through a callus phase, lacked observed somaclonal variation in phenotype. Rogers (1993b) has shown that *H. retusa, H. emelae*, *H. mirablis* and *H. mutica* can be micropropagated and hardened-off within 16 weeks using the organogenic route of differentiation, and the numbers of plants which can be regenerated per explant is comparable to the results obtained in the present study. Organogenesis, therefore, seems to be a feasible route for plant production.

Although in this study, a treatment was established for the reversal of vitrification i.e. incubation of vitrified plantlets with containers of silica gel, this method was extremely labour-intensive as the silica gel had to be replaced every 24 hours. A less laborious means of reducing the relative humidity in the culture vessel is required. One possible approach, not tried in this study, would be the testing of the various treatments at the onset of culture initiation to avoid, rather than reverse, the development of this condition. A potential disadvantage of this strategy is that the some of the treatments to be tested, could affect the high propagation rates.

Although many plantlets can be produced by embryogenesis (or organogenesis, Rogers, 1993b) thereby facilitating substantial return of plants to conserved areas, cryopreservation provides a storage strategy for conservation. This technique involves the freezing of various plant tissues, including somatic embryos and shoot meristems, to low temperatures (-80 °C to -196 °C), and subsequent thawing and plant regeneration (Krogstrup *et al.*, 1992). Cryopreserved material is thought to remain genetically stable thus minimising genetic drift and other genotypic changes which can occur (Withers, 1980, 1982, 1985, 1988).

Chapter 4: ESTABLISHMENT OF STERILISATION AND REGENERATION PROTOCOLS FOR *Cryptocarya latifolia*

4.1. INTRODUCTION

4.1.1. The botany of *Cryptocarya latifolia*

The genus *Cryptocarya* (Family: Lauraceae) is represented by six species in southern Africa. Of these, three (*C. latifolia*, *C. myrtifolia* and *C. woodii*) are restricted to the coastal regions of the eastern Cape, Transkei and KwaZulu/Natal (Coates-Palgraves, 1981). *Cryptocarya latifolia* (Plate 4.1) has a wide range of uses (including medicinal, mythological and structural) and wild population numbers have been substantially diminished as a result of over-exploitation (Cunningham, 1988).

Cryptocarya is a tall forest tree found primarily along streams and rivers. According to Pooley (1993) the bark is smooth, grey, brown and the stem coppices readily. The leaves are alternate, smooth, leathery and dark green above, bluish-green beneath. The midrib and veins are prominent and are covered with coppery hairs. The leaves are 3-veined at the base, the margins wavy and the stalks hairy. Leaf tips may be rounded or notched. Young leaves and stems are covered in hairs. The flowers appear in small sprays (June - September) with the round fruit ripening black after shedding (October - December). The fruits are eaten by birds and antelope, and butterflies of the genus *Charaxes* are often found associated with this tree.

Plate 4.1: *Cryptocarya latifolia*

A young specimen (approximately five years old) (bar = 150 mm)

4.1.2. The uses of *C. latifolia* in South Africa

According to Cunningham (1988) many rural communities rely on indigenous plants as sources of fuel, shelter, food and medicines. The genus *Cryptocarya* is particularly important in all of these respects. The bark, in combination with crocodile fat is used to treat chest complaints (Pooley, 1993) and is also used as an alternative to that of the rare Black Stinkwood, *Ocotea bullata*, for colds and influenza (Nichols pers. comm.). The wood of *C. latifolia* is also heavily utilised in the making of furniture (Coates-Palgraves, 1981).

4.1.3. Previous studies on the genus *Cryptocarya*

There have been no reported studies on the micropropagation of *Cryptocarya* spp. to date. Much of the work that has been undertaken within this genus has been involved with the isolation of organic constituents from either the bark, wood or leaves (Fu *et al.*, 1993; Shoei-Sheng *et al.*, 1993), many of which have been found to be biologically active.

4.1.4. Published reports on the development of micropropagation protocols for woody species

In comparison to the success achieved in establishing micropropagation protocols for herbaceous plants, little progress has been made with regard to the *in vitro* regeneration of woody species. However, protocols have been developed whereby shoots or rooted shoots are produced via indirect or direct organogenic routes (Table 4.1). Furthermore, whole plants have also been regenerated via somatic embryos (Table 4.2). There is a high degree of variability of the nutrient formulation used for

different classes of plants (e.g. bulbous, woody, succulents), the most commonly used nutrient media being MS (Murashige and Skoog, 1962), Woody Plant Medium (WPM) (Lloyd and McCown, 1980) and B5 (Gamborg *et al.*, 1968).

Table 4.1: Some of the reported studies of micropropagation via organogenesis in woody dicotyledonous species.

Species	Reference
Acacia albida	Duhoux and Davies (1985)
Acacia koa	Skolmen and Mapes (1978)
Acacia liglata	Williams *et al.* (1985)
Albizia rebeck	Gharyal and Maheshwari (1983)
Alnus glutinosa	Garton *et al.* (1981)
Araucaria cunninghamii	Haines and de Fossard (1977)
Betula spp.	McCown and Amos (1979); Minocha (1980); Srivastava and Steinhauer (1981)
Bougainvillea glabra	Sharma *et al.* (1980)
Citrus spp.	Chaturvedi and Mitra (1974); Kitto and Young (1981)
Coffea arabica	Herman and Haas (1975); Kartha *et al.* (1981); Sondahl *et al.* (1984)
Commiphora wightii	Barve and Mehta (1993)
Cryptomeria japonica	Isikawa (1974); Wang (1978)
Eucalyptus spp.	de Fossard *et al.* (1974); Harkney and Barker (1980); Gupta *et al.* (1981); McComb and Bennett (1982)
Ficus benjamina	Makino *et al.* (1977)
Kalmia latifolia	Lloyd and McCown (1980)
Lonicera japonica	Georges *et al.* (1993)
Malus spp.	Lundergan and Janick (1979); Mehra and Sachdeva (1979); Nemeth (1979); Werner and Boe (1980); Liu *et al.* (1983)
Picea spp.	De Torok and Thimann (1961); Huhtinen (1976); Webb and Street (1977); Bornman (1983)
Pinus spp.	Webb and Street (1977); David *et al.* (1982); Rancillac *et al.* (1982)
Populus spp.	Whitehead and Giles (1977); Chalupa (1979); Mehra and Cheema (1980); Chang (1981)
Prunus spp.	Boxus (1975); Tabak and Kesler (1977); Jones and Hopgood (1979); Lane (1979); Skirvin and Rukan (1979); Skirvin *et al.* (1980); Rugini and Verma (1982, 1983); Snir (1983)
Pseudotseuga menziesii	Winton and Verhagen (1977)
Quercus robur	Chalupa (1984)
Ribes spp.	Harvey and Grasham (1977)
Salix spp.	Bhojwani (1980); Chalupa (1983)
Syringa vulgaris	Hildebrandt and Harney (1983)
Tectona grandis	Gupta *et al.* (1980)
Ulmus spp.	Chalupa (1979); Ulrich *et al.* (1984)

Table 4.2: Some of the reported studies of somatic embryogenesis in woody dicotyledonous species. DSE: direct somatic embryogensis; ISE: indirect somatic embryogenesis.

Species	DSE/ISE	Reference
Actinidia chinensis	ISE	Harada (1975)
Acer negundo	DSE	Radojevic (1980, 1988)
Albizia lebeck	DSE	Gharyal and Maheshwari (1981)
Betula pendula	ISE	Kurten *et al.* (1990); Nuutila *et al.* (1991)
Carica spp.	ISE	Litz and Conover (1978, 1980, 1981, 1982, 1983); Moore and Litz (1984); Jordan *et al.* (1983)
Carya illinoinensis	DSE	Hansen and Lazarte (1982, 1984)
Castanea spp.	DSE	Gonzalez *et al.* (1985)
Citrus spp.	DSE/ISE	Rangaswamy (1961); Rangan *et al.* (1968); Button and Bornman (1971); Kochba *et al.* (1972); Esan (1973); Button and Botha (1975); Rajbhansali and Arya (1979); Mitra and Chaturvedi (1982); Navarro *et al.* (1985); Gmitter and Moore (1986); Zhan-ao *et al.* (1990); Gmitter and Ling (1991)
Coffea spp.	DSE	Staritsky (1970); Sondahl and Sharp (1977); Sondahl *et al.* (1984)
Corylus aveliana	DSE	Radovejic *et al.* (1985); Perez *et al.* (1986)
Eucalyptus spp.	DSE/ISE	Watt *et al.* (1991)
Eugenia spp.	DSE	Litz (1984)
Ilex aquifolium	DSE	Hu and Sussex (1972); Hu *et al.* (1978)
Juglans spp.	DSE	Tulecke and McGranahan (1985)
Lavandula augustifolia	ISE	Quazi (1980)
Liquidambar styraciflua	ISE	Sommer and Brown (1980)
Liriodendron tulipifera	ISE	Merkle and Sommer (1986)
Malus spp.	DSE/ISE	Eicholtz *et al.* (1979); Liu *et al.* (1983); James *et al.* (1984); Mehra and Sachdeva (1984)
Mangifera indica	DSE	Litz *et al.* (1984)
Myrciaria cauliflora	DSE	Litz (1984)
Ocotea catharinensis	DSE	Moura-Costa *et al.* (1993)
Paulownia tomentosa	ISE	Radovejic (1979)
Persea americana	ISE	Mooney and van Staden (1987)
Prunus hybrid	ISE	Jones *et al.* (1984)
Pterocarya spp.	DSE	Mehra and Mehra (1971); Tulecke and McGranahan (1985)
Pyrus spp.	DSE	Mehra and Jaidka (1980); Janick (1982)
Quercus serris	DSE	Ostrolucka and Pretova (1991)
Ribes rubrum	DSE	Zatkyo *et al.* (1975)
Robinia pseudoacacia	DSE	Merkle and Wiecko (1989)
Rosa hybrida	ISE	Noriega and Sondahl (1991)
Santalum album	ISE	Rao (1985); Bapat and Rao (1979); Rao and Bapat (1980); Lakshmi *et al.* (1979)
Sapindus trifoliatus	ISE	Desai *et al.* (1987)
Simmondsia chinensis	DSE	Lee and Thomas (1985); Wang and Janick (1986)
Theobroma cacao	DSE	Pence *et al.* (1979, 1980); Kononowicz and Janick (1988)
Vitis vinifera	ISE	Krul and Worley (1977); Coutos-Thevenot *et al.* (1991)

4.2. MATERIALS AND METHODS

4.2.1. Establishment of sterile explants

4.2.1.1. Preliminary tests for contaminants

Leaf explants and nodal segments (Plate 4.2) were placed onto solid MS (Murashige and Skoog, 1962) nutrient medium supplemented with 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar and placed into culture at 25 °C in the light (16:8 hour photoperiod, 200 $\mu Em^{-2}s^{-1}$). The extent of contamination was recorded after five days in culture.

4.2.1.2. Initial sterilisation procedure

Branches from young trees were cut from field-grown plants. Stem segments of approximately 200 mm in length, were cut and dipped in 70% [v/v] ethanol for 2 - 3 minutes. Subsequently, the stems were transferred to a 1% [v/v] solution of sodium hypochlorite containing five drops of Tween-20 for 15 - 20 minutes. After thorough washing with sterile water, the leaves were cut into squares approximately 10 mm^2 and plated on to solid MS medium supplemented with 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. Nodal segments consisting of 20 - 30 mm of stem and a portion of the leaf (approximately the basal third), were plated onto the same medium in culture tubes. Cultures were screened for the presence of contaminants after 5 days at 25 °C, 16:8 hour light:dark photoperiod.

4.2.1.3. The use of anti-fungal agents

Explants were sterilised as described in Section 4.2.1.2. Several fungicides were tested for efficacy against fungal contaminants in both leaf and nodal segment cultures (Table 4.3).

Table 4.3: Details of the fungicides used to determine the most efficient for use in *Cryptocarya* cultures

Fungicide/Fungicide combinations	Active ingredient	Concentration at which tested ($g.l^{-1}$)
Benlate (Effecto SA)	benomyl	0.1 1.0
Previcur N (FBC Holdings)	probamacarb hydrochloride	0.36
Amphotericin B (Boeringer)	Amphotericin B	0.0025
Bravo (Shell SA (Pty) Ltd)	chlorothalonil	0.25 0.5
Benlate : Previcur N	benomyl : probamacarb hydrochloride	0.1 : 0.36 1.0 : 0.36
Benlate : Amphotericin B	benomyl : Amphotericin B	0.1 : 0.0025 1.0 : 0.0025
Benlate : Bravo	benomyl : chlorothalonil	0.1 : 0.25 1.0 : 0.25

Explants were either plated directly on to MS nutrient medium or were subjected to a wash solution for varying lengths of time (1, 3, 6, 12, 24 and 30 hours) before culture initiation. The wash solution was a liquid MS nutrient medium supplemented with 30 $g.l^{-1}$ sucrose, 0.01 $g.l^{-1}$ ascorbic and citric acids, and the fungicides as described in Table 4.3. The presence of fungal contamination was recorded after 5 days in the light at 25 °C (200 $\mu Em^{-2}s^{-1}$).

4.2.1.4. Control of phenolic exudation

Within four days in culture, the presence of phenolic compounds was observed in the medium. These compounds are leached into the medium surrounding the explant as a wound response and eventually are associated with decay and death of the explant. Polyvinylpyrollidone (0.05 $g.l^{-1}$) and ascorbic (0.01 $g.l^{-1}$) and citric acids (0.01 $g.l^{-1}$) were tested for the ability to scavenge phenolic compounds in the culture medium, ascorbic and citric acids being tested in combination. These constituents were added to either the nutrient medium alone or were added to both the wash solution (Section 4.2.1.3.) and the nutrient medium. In addition, the cultures were placed in the dark for 5 days to prevent phenolic compounds from leaching into the culture medium.

4.2.1.5. Final sterilisation protocol

Stem segments were collected from field-grown material and dipped into 70% [v/v] ethanol for 10 - 20 seconds. The plant material was then transferred to a 1% [v/v] NaOCl solution (containing 5 drops Tween-20) for 20 - 25 minutes. The stems and leaves were rinsed three times with sterile water and were transferred to MS nutrient

solution supplemented with 30g.l^{-1} sucrose, 10 g.l^{-1} agar, 1 g.l^{-1} Benlate, 0.0025 g.l^{-1} Amphotericin B, 0.01 g.l^{-1} ascorbic acid and 0.01 g.l^{-1} citric acid.

4.2.2. Development of micropropagation protocols

4.2.2.1. Release of dormant axillary buds

Induction of axillary budding provides a large amount of pathogen-free material for subsequent shoot multiplication, elongation and rooting of multiplied shoots. Several media formulations and hormone combinations have been developed for both crop and ornamental woody species. In this investigation, several different media formulations (full-strength MS, half-strength MS and WPM) and plant growth regulator combinations were tested for the induction of axillary buds from nodal segments (Table 4.4). Explants with dormant buds (Plate 4.2 and Figure 4.1) were cultured onto media solidified with either 10 g.l^{-1} agar or 4 g.l^{-1} Gelrite in culture tubes.

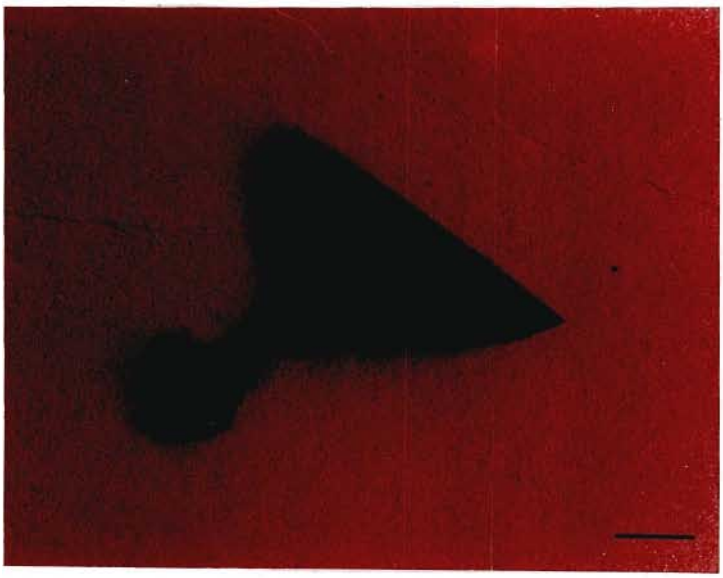


Plate 4.2: Typical nodal segment explant used to induce axillary budding. Explant cultured on MS medium supplemented with 30 g.l^{-1} sucrose, 10 g.l^{-1} agar, 0.01 g.l^{-1} ascorbic acid, 0.01 g.l^{-1} citric acid, 0.1 g.l^{-1} Benlate, 0.0025 g.l^{-1} Amphotericin B, 0.01 g.l^{-1} biotin, 0.01 g.l^{-1} calcium pantothenate, 1.0 mg.l^{-1} BAP and 0.01 mg.l^{-1} NAA. (bar = 4 mm)

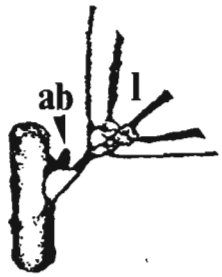


Figure 4.1: Representation of a typical nodal segment explant showing dormant bud (ab) and attached leaf (l)

Table 4.4: Plant growth regulator combinations tested for the induction of axillary budding in nodal explants. Explants were cultured onto either full strength MS nutrients, half strength MS nutrients or WPM nutrients after washing. All media were supplemented with 0.1 $g.l^{-1}$ Benlate, 0.0025 $g.l^{-1}$ Amphotericin B, 0.01 $g.l^{-1}$ ascorbic acid, 0.01 $g.l^{-1}$ citric acid, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. *: 0.001 $g.l^{-1}$ biotin and 0.001 $g.l^{-1}$ calcium pantothenate.

PGRs ($mg.l^{-1}$)	other ($mg.l^{-1}$)
(1) BAP + (0.1) GA_3	
(5.0) BAP + (0.01) GA_3	
(5.0) BAP + (0.1) GA_3	
(11.625) BAP + (0.002) NAA	
(0.05) BAP + (0.1) IAA	
(0.05) BAP + (0.5) IAA	
(0.2) BAP + (1.0) IAA	
(0.05) BAP + (2.0) IAA	
(5) BAP + (0.5) NAA	*
(1) BAP + (0.1) NAA	*
(1) BAP + (0.01) NAA	*
(0.2) BAP + (0.1) NAA	*
(0.2) BAP + (0.01) NAA	*
(0.5) BAP + 0.01) NAA	*

4.2.2.2. Induction of shoot multiplication

The young shoots (see Section 4.2.2.1) were excised from the initial explants after three to four weeks, transferred to culture tubes and cultured onto three different nutrient media (full strength MS, half strength MS and WPM). Each nutrient formulation was supplemented with 30 $g.l^{-1}$ sucrose, 10$g.l^{-1}$ agar, 0.01 $g.l^{-1}$ biotin and 0.01 $g.l^{-1}$ calcium pantothenate and contained different growth regulator combinations (0.2 - 1.0 $mg.l^{-1}$ BAP and 0.01 - 0.1 $mg.l^{-1}$ NAA). The explants were incubated at 25 °C in a 16:8 hour photoperiod (200 $\mu Em^{-2}s^{-1}$) for three to four weeks. Proliferation of axillary buds was recorded.

4.2.2.3. Establishment of callus cultures

Using leaf explants (cultured in Petri dishes) from young leaves obtained from field-grown plants, several hormone combinations were tested for callus initiation (Table 4.5). Explants were plated out in Petri dishes containing MS nutrient medium supplemented with 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. The efficacy of these media for callus induction was assessed after 5 weeks in culture. Hormone combinations known to induce organogenic and embryogenic callus were tested in this investigation. The leaves were divided into several regions (Figure 4.2) to determine which region was found to enhance callus production.

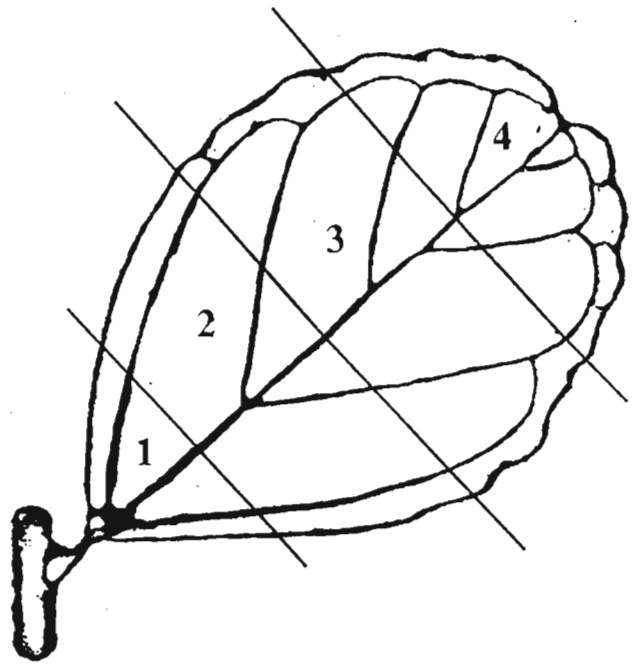


Figure 4.2: The four different regions of the leaf tested to determine which part of the leaf was the best explant to use for callus production.

Table 4.5: The plant growth regulator combinations used to initiate callus from leaf explants of 5 year-old *Cryptocarya latifolia* trees. Explants were cultured onto MS nutrient medium containing 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar.

Plant Growth regulators ($mg.l^{-1}$)
(1)2,4-D
(3) 2,4-D
(5) 2,4-D
(1) 2,4-D + (0.2) Kinetin
(3) 2,4-D + (0.2) Kinetin
(5) 2,4-D + (0.2) Kinetin
(1) 2,4-D + (0.5) Kinetin
(3) 2,4-D + (0.5) Kinetin
(5) 2,4-D + (0.5) Kinetin
(1) 2,4-D + (1.0) Kinetin
(3) 2,4-D + (1.0) Kinetin
(5) 2,4-D + (1.0) Kinetin
(0.1) NAA + (0.01) BAP
(0.1) NAA + (1.0) BAP
(0.5) NAA + (0.01) BAP
(0.5) NAA + (1.0) BAP

4.2.3. Photography

Stages of axillary bud induction, shoot multiplication and callus formation were recorded using a Nikon FM2 camera with a 60 mm Mikro Nikkor macro lens.

4.2.4. Analysis of data

The extent of contamination, axillary bud induction, shoot multiplication and callus formation was recorded. Experiments were repeated three times with the number of explants ranging between thirty and fifty for each treatment. Where appropriate, differences in the mean percentages of recorded parameters were assessed using one-way ANOVA (Statgraphics Plus, 1993). Alphabetical values were assigned to the means obtained for each treatment, and are significantly different if they do not share the same letter.

4.3. RESULTS

4.3.1. Generation of aseptic explants

4.3.1.1. Initial tests for contaminants

Preliminary investigations using field-grown material of *C. latifolia* were carried out to investigate the level of fungal contamination and the extent of phenolic production from both leaf and nodal segment explants as described in Section 4.2.1. Fungal contamination ranged from 40% to 100% depending on the treatments used (Table 4.6A and 4.6B and 4.7A and 4.7B). Fungal contamination was highest (98% and 100% for nodal segment and leaf explants respectively) when the sterilisation time in sodium hypochlorite was 10 minutes and the explants were washed in ethanol for 10 or 30 seconds. Fungal contamination decreased when the explants were washed in ethanol for longer periods of time. Contamination was reduced to 25% (nodal segment) and 40% (leaf) when explants were washed in ethanol for 5 minutes and then transferred to sodium hypochlorite for 30 minutes. However, when placed into culture, explant tissue appeared dehydrated and extensive tissue browning occurred. Although there was a high (65%) degree of contamination when explants were washed for 30 seconds in ethanol and 25 minutes in sodium hypochlorite, explant tissue appeared healthy. The results of these tests are illustrated in Table 4.6A and 4.6B). The contamination observed may have been attributed to the presence of endogenous fungi and not surface contaminants. Several fungicides were included in the sterilisation protocol in order to eliminate the contaminants.

In addition to fungal contaminants, phenolic exudation into the surrounding medium was extremely high (90%-100%) for all treatments. Polyvinylpyrollidone and ascorbic and citric acids were tested as they are known antioxidants (Figure 4.3).

Table 4.6A: The effect of ethanol (70% [v/v]) pre-treatment on fungal contamination in nodal segment explants. The explants were pre-treated in ethanol for varying lengths of time and then soaked in 1% [v/v] NaOCl for either 10, 15, 20, 25 or 30 minutes. n (number of explants) = 30 per treatment.

Time in 70% ethanol (seconds)	Time in 1% NaOCl	% Contamination
10	10	98[k]
	15	95[j]
	20	80[g]
	25	75[f]
	30	65[d]
30	10	98[k]
	15	90[i]
	20	80[g]
	25	65[d]
	30	60[c]
60	10	90[i]
	15	85[h]
	20	85[h]
	25	70[e]
	30	60[c]
300	10	80[g]
	15	80[g]
	20	70[e]
	25	50[b]
	30	25[a]

Table 4.6B: The effect of ethanol (70% [v/v]) pre-treatment on fungal contamination in leaf explants. The explants were pre-treated in ethanol for varying lengths of time and then soaked in 1% [v/v] NaOCl for either 10, 15, 20, 25 or 30 minutes. n (number of explants) = 30 per treatment.

Time in 70% ethanol (seconds)	Time in 1% NaOCl	% Contamination
10	10	100[j]
	15	99[i]
	20	95[h]
	25	80[e]
	30	75[d]
30	10	100[j]
	15	95[h]
	20	85[f]
	25	65[b]
	30	65[b]
60	10	95[h]
	15	85[f]
	20	80[e]
	25	80[e]
	30	70[c]
300	10	90[g]
	15	90[g]
	20	70[c]
	25	65[b]
	30	40[a]

4.3.1.2. The effect of anti-fungal agents on fungal contamination

As a result of the high levels of fungal contaminants (Tables 4.6A and 4.6B), the pre-treatment was modified to include a wash solution (30 hour wash) containing appropriate nutrients and supplemented with various combinations of fungicides (Table 4.3). The results of this investigation (Tables 4.7A and 4.7B) revealed that an increased (1 $g.l^{-1}$) concentration of Benlate was required in the wash solution to reduce fungal infection in culture. However, this concentration was lethal to explants in culture and was reduced to 0.1 $g.l^{-1}$. Combinations of Bravo and Previcur N with either 0.1 $g.l^{-1}$ or 1 $g.l^{-1}$ Benlate did not have any enhanced effect in reducing fungal contaminants in either leaf or nodal segment cultures. In contrast, a combination of Amphotericin B and Benlate reduced fungal contamination to approximately 10 % compared with the previous value of 65% (Tables 4.7A and 4.7B).

4.3.1.3. The effect of anti-oxidants on contamination by phenolic compounds

Both leaf and nodal segment cultures were contaminated with phenolic compound exudatives after 5 days in culture. A period in the dark did not reduce the extent of phenolic exudation significantly (contamination remained at 60 - 65%). Based on these observations, it was decided to test ascorbic and citric acids and polyvinylpyrollidone (Figure 4.3) as they are known to be antioxidants. A mixture of ascorbic (0.1 $g.l^{-1}$) and citric acid (0.1 $g.l^{-1}$) reduced phenolic exudation by 50% when incorporated into the culture medium. If the acids were added to the 30 hour wash (Section 4.1.2.3) solution, phenolic exudation was eliminated completely. Polyvinylpyrollidone had little effect on the reduction of phenolic production.

Table 4.7A: The effect of different fungicides and fungicide combinations on fungal contamination in nodal segment explants. Explant material was washed in a nutrient solution (Section 4.1.2.3) containing the fungicide combinations for varying lengths of time (1 - 30 hours) and then transferred to a culture medium containing the fungicides as shown below.

Fungicide Treatment		Time in wash (hours)	% contamination
Type	Concentration ($g.l^{-1}$)		
Benlate	0.1	1	65^{l}
		3	65^{l}
		6	65^{l}
		12	65^{l}
		24	55^{j}
		30	50^{i}
	1.0	1	55^{j}
		3	55^{j}
		6	50^{i}
		12	50^{i}
		24	30^{e}
		30	15^{c}
Previcur N	0.36	1	65^{l}
		3	65^{l}
		6	65^{l}
		12	65^{l}
		24	65^{l}
		30	60^{k}
Benlate : Previcur N	0.1 : 0.36	1	65^{l}
		3	65^{l}
		6	65^{l}
		12	75^{n}
		24	80^{o}
		30	80^{o}
	1.0 : 0.36	1	65^{l}
		3	65^{l}
		6	65^{l}
		12	65^{l}
		24	75^{n}
		30	75^{n}
Bravo	0.25	1	65^{l}
		3	65^{l}
		6	70^{m}
		12	75^{n}
		24	75^{n}
		30	88^{p}
	0.50	1	65^{l}
		3	65^{l}
		6	65^{l}
		12	70^{m}
		24	70^{m}
		30	70^{m}
Amphotericin B	0.0025	1	65^{l}
		3	65^{l}

Table 4.7B: The effect of different fungicides and fungicide combinations on fungal contamination in leaf explants. Explant material was washed in a nutrient solution (Section 4.1.2.3) containing the fungicide combinations for varying lengths of time (1 - 30 hours) and then transferred to a culture medium containing the fungicides as shown below.

Fungicide Treatment		Time in wash (hours)	% contamination
Type	Concentration ($g.l^{-1}$)		
Benlate	0.1	1	65^{k}
		3	65^{k}
		6	65^{k}
		12	65^{k}
		24	65^{k}
		30	50^{h}
	1.0	1	65^{k}
		3	65^{k}
		6	55^{i}
		12	50^{h}
		24	45^{g}
		30	45^{g}
Previcur N	0.36	1	65^{k}
		3	65^{k}
		6	65^{k}
		12	65^{k}
		24	65^{k}
		30	65^{k}
Benlate : Previcur N	0.1 : 0.36	1	65^{k}
		3	65^{k}
		6	75^{m}
		12	85^{o}
		24	85^{o}
		30	90^{p}
	1.0 : 0.36	1	65^{k}
		3	70^{l}
		6	70^{l}
		12	80^{n}
		24	85^{o}
		30	85^{o}
Bravo	0.25	1	65^{k}
		3	65^{k}
		6	75^{m}
		12	85^{o}
		24	90^{p}
		30	90^{p}
	0.50	1	65^{k}
		3	65^{k}
		6	70^{l}
		12	75^{m}
		24	75^{m}
		30	75^{m}
Amphotericin B	0.0025	1	65^{k}
		3	65^{k}
		6	65^{k}

Benlate : Amphotericin B	0.1 : 0.0025	1	65^{k}
		3	45^{g}
		6	45^{g}
		12	65^{k}
		24	20^{d}
		30	5^{a}
	1.0 : 0.0025	1	65^{k}
		3	40^{f}
		6	25^{e}
		12	15^{c}
		24	10^{b}
		30	5^{a}
Benlate : Bravo	0.1 : 0.25	1	65^{k}
		3	60^{j}
		6	60^{j}
		12	55^{i}
		24	50^{h}
		30	50^{h}
	1.0 : 0.25	1	65^{k}
		3	65^{k}
		6	55^{i}
		12	50^{h}
		24	50^{h}
		30	50^{h}

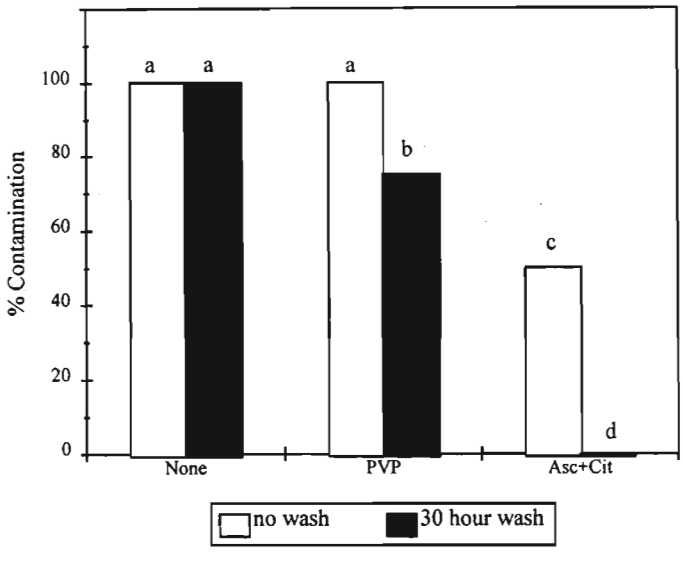


Figure 4.3: The effect of selected antioxidants on the release of phenolic compounds into the culture medium. Explant material was placed directly into culture on a nutrient medium containing MS nutrients, 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar, 1 $g.l^{-1}$ Benlate, 0.0025 $g.l^{-1}$ Amphotericin B and the antioxidant combinations tested or were washed for 30 hours in a wash solution (Section 4.1.2.3) containing the antioxidants and then placed into culture.

None: no antioxidants in culture medium or wash solution; PVP: 0.05 $mg.l^{-1}$ polyvinylpyrollidone;

Asc+Cit: 0.01 $mg.l^{-1}$ ascorbic acid and 0.01 $mg.l^{-1}$ citric acid. n (number of explants) = 30 per treatment.

4.3.1.4. Final sterilisation procedure

Stems segments, 200 mm to 300 mm long, were washed in 70% [v/v] ethanol for 30 seconds after which they were transferred to 1% [v/v] sodium hypochlorite solution for 20 minutes. The stems were then rinsed three times with sterile water under sterile conditions, and placed in a nutrient-rich wash solution (Section 4.1.2.3). The plant material was gently agitated on an orbital shaker for 30 hours. The wash solution was supplemented with 1 $g.l^{-1}$ Benlate, 0.0025 $g.l^{-1}$ Amphotericin B and 0.1 $g.l^{-1}$ each of ascorbic and citric acids. Subsequent to washing, explants were placed onto the appropriate medium (Section 4.2.1) supplemented with the above additives. Benlate was used at a concentration of 0.1 $g.l^{-1}$ in the culture medium.

4.3.2. Establishment of micropropagation protocols

4.3.2.1. Initiation of axillary bud growth

The extent of shoot production on the three different media tested is summarised in Table 4.8. The use of MS nutrient medium resulted in the greatest number of nodal explants producing shoots. One shoot was produced in each axil after three to five days in culture (Plate 4.3A). After three to four weeks, the leaves had expanded (Plate 4.3B) and shoots were excised from the original explants and transferred to another culture medium to induce shoot multiplication (Plate 4.3C).

Table 4.8: Effect of nutrient medium and plant growth regulators on emergence of axillary buds and senescence in nodal explants. Tested media: A: MS; B: ½MS; C: WPM.. Each treatment was repeated three times; n= 50 per treatment. *: 0.1 $mg.l^{-1}$ Biotin and 0.1 $mg.l^{-1}$ Ca pantothenate added to culture medium. n (number of explants) = 30 per treatment.

		A		B		C	
PGRs ($mg.l^{-1}$)	other ($mg.l^{-1}$)	senescence (%)	explants with shoots (%)	senescence (%)	explants with shoots (%)	senescence (%)	explants with shoots (%)
(1) BAP + (0.1) GA_3		50	40^{l}	55	40^{l}	65	40^{l}
(5.0) BAP + (0.01) GA_3		30	65^{m}	30	60^{l}	55	60^{l}
(5.0) BAP + (0.1) GA_3		30	70^{n}	20	55^{k}	30	60^{l}
(11.625) BAP + (0.002) NAA		80	15^{d}	75	15^{d}	35	20^{e}
(0.05) BAP + (0.1) IAA		30	10^{c}	30	5^{b}	85	5^{b}
(0.05) BAP + (0.5) IAA		20	60^{l}	20	40^{i}	20	35^{h}
(0.2) BAP + (1.0) IAA		30	0^{a}	30	0^{a}	35	0^{a}
(0.05) BAP + (2.0) IAA		0	50^{j}	10	30^{g}	35	35^{h}
(5) BAP + (0.5) NAA	*	0	50^{j}	10	30^{g}	0	25^{f}
(1) BAP + (0.1) NAA	*	10	50j	25	35^{h}	15	25^{f}
(1) BAP + (0.01) NAA	*	0	100^{p}	15	80^{o}	25	60^{l}
(0.2) BAP + (0.1) NAA	*	20	30^{g}	20	30^{g}	20	20^{e}
(0.2) BAP + (0.01) NAA	*	50	80^{l}	65	25^{f}	80	20^{e}
(0.5) BAP + 0.01) NAA	*	20	35^{h}	30	20^{e}	45	15^{d}

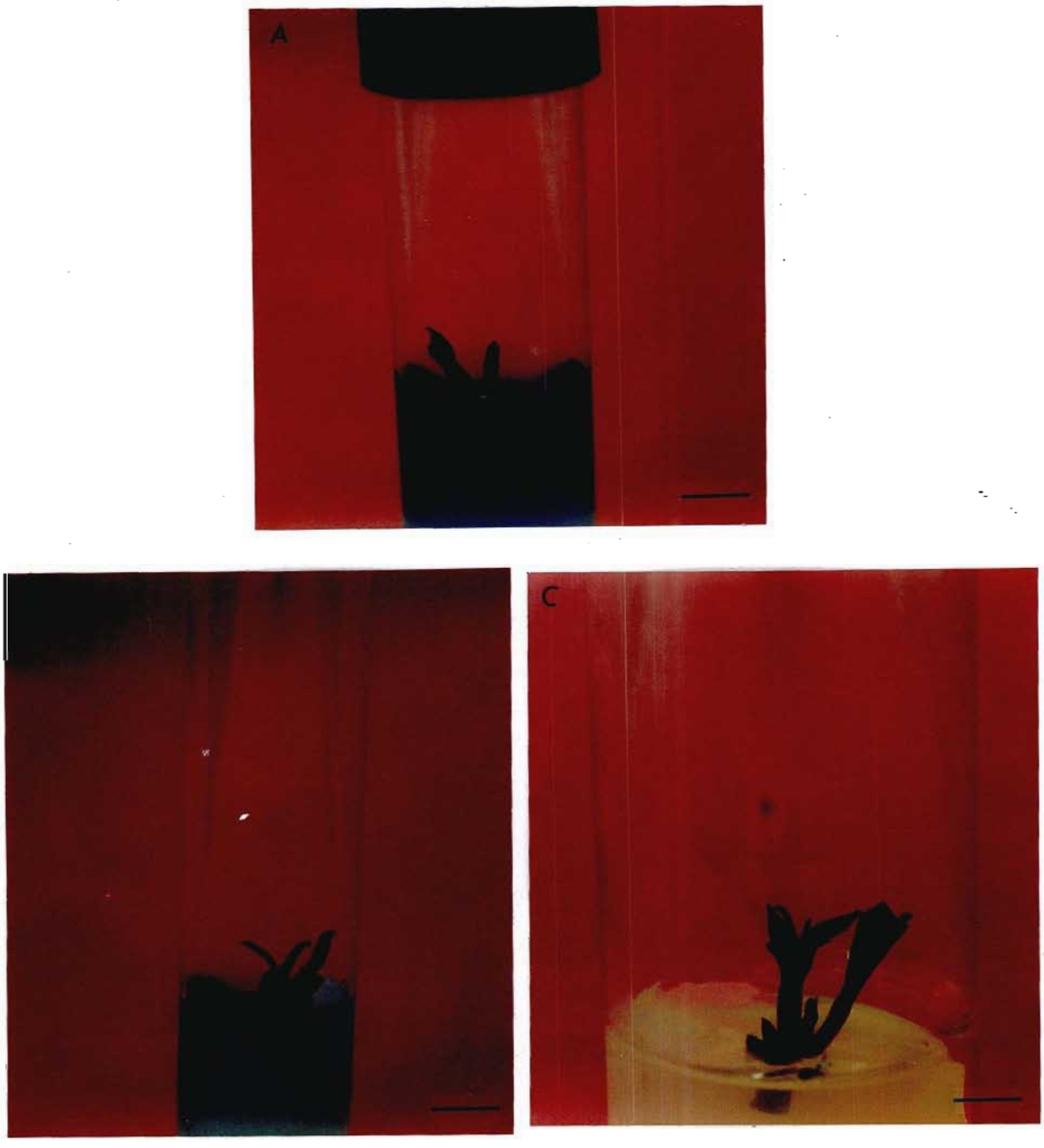


Plate 4.3: Axillary bud growth and shoot proliferation in *C. latifolia*. Explants were cultured on MS medium supplemented with 0.1 $mg.l^{-1}$ Biotin, 0.1 $mg.l^{-1}$ Calcium pantothenate, 0.01 $mg.l^{-1}$ NAA, 1 $mg.l^{-1}$ BAP, 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar , 0.1 $g.l^{-1}$ Benlate and 0.0025 $g.l^{-1}$ Amphotericin B.

A: Induction of axillary budding (bar = 10 mm)

B: Shoot extension and unfurling of leaves (bar = 10 mm)

C: Axillary bud/shoot multiplication (bar = 20 mm)

4.3.2.2. Effect of growth regulators on shoot multiplication

Shoots produced (see 4.3.2.1) were excised from the axils of nodal segments and used to test different hormone combinations for the induction of shoot multiplication (Table 4.9). The hormone combination inducing 100% axillary budding (1 $mg.l^{-1}$ BAP and 0.01 $mg.l^{-1}$ NAA) was also found to induce axillary bud proliferation after two weeks in culture.

Table 4.9: Effect of plant growth regulators on shoot multiplication in *Cryptocarya latifolia*. Explants were cultured on MS nutrient medium supplemented with 0.1 $g.l^{-1}$ Biotin, 0.1 $g.l^{-1}$ Calcium pantothenate, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar. Each treatment was repeated three times; n (number of explants) = 50 per treatment.

PGRs ($mg.l^{-1}$)	Explants with >1 shoot (%)	Number of shoots/ explant
(0.2) BAP + (0.1) NAA	0	-
(1.0) BAP + (0.1) NAA	10	1
(0.2) BAP + (0.01) NAA	0	-
(0.5) BAP + (0.01) NAA	0	-
(1.0) BAP + (0.01) NAA	60	4

4.3.2.3. Effect of gelling agent on explant establishment, axillary bud induction and shoot multiplication

Gelrite, as an alternative gelling agent to agar, has been found to increase multiplication rates in other woody species (Blakeway, 1991; MacRae and van Staden, 1990;). In this study, shoots were cultured onto MS medium containing 30 $g.l^{-1}$ sucrose, 0.1 $mg.l^{-1}$ biotin, 0.1 $mg.l^{-1}$ calcium pantothenate and either 10 $g.l^{-1}$ agar or 4 $g.l^{-1}$ Gelrite. After two weeks, no multiplication had occurred in explants on the

medium solidified with Gelrite. Furthermore, the shoots became brown and after three weeks, appeared dead.

4.3.2.4. Initiation of callus

Using young leaf material obtained from field-grown trees, 33% of the explants produced calli on MS nutrient medium containing 3 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ kinetin after 6 - 8 weeks in culture. Callus formed on explants derived from the petiolar region (Plate 4.4A) only. No callus was formed on explants from regions 2, 3 and 4 (Section 4.2.2.3). Microscopic examination of the callus revealed that the callus was non-embryogenic (Plate 4.4B).

Plate 4.4: Callus formation from *C. latifolia* leaf explants

A: Callus development on the petiolar region of the leaf. Leaf material was cultured on medium containing MS nutrients, 3 $mg.l^{-1}$ 2,4-D, 0.2 $mg.l^{-1}$ kinetin and 10 $g.l^{-1}$ agar. (bar = 4 mm)

B: Non-embryogenic cells from callus formed from the petiolar region of the leaf. (Bar = 0.15 mm)

4.4. DISCUSSION

4.4.1. Culture establishment

Before *in vitro* regeneration protocols can be established, the plant material being used must be free from pathogens. A large portion of the time expended using *Cryptocarya latifolia*, was devoted to the establishment of an effective sterilisation protocol using both leaf and nodal segment explants. Fungal contamination posed a major problem in this study (Table 4.6). The reason for this may be that the explant material was obtained from field-grown plants and, often, fungal contamination is a particular problem when the parent plant is from a warm, sub-tropical climate as is the case with *Cryptocarya latifolia*. Woody species have been found to be particularly contaminated with micro-organisms and the systemic pathogens which are frequently encountered cannot be removed by conventional surface sterilisation procedures (Young *et al.*, 1984; Constantine, 1986; Warrag *et al.*, 1990; Hussein *et al.*, 1994).

In this investigation, several fungicides and combinations of fungicides were tested for their efficacy against fungal contaminants. A combination of Benlate (1.0 $g.l^{-1}$ in the wash solution (Section 4.1.2.3) and 0.1 $g.l^{-1}$ added to the culture medium) and Amphotericin B (0.0025 $g.l^{-1}$) was found to reduce fungal contamination significantly (Table 4.7A and 4.7B). Benlate acts by interfering with fungal microtubules (Shields *et al.*, 1984) and inhibits spore germination (Hassall, 1990). It is commonly used as a systemic fungicide on crop species but recently, it has been claimed that certain formulations of this fungicide are phytotoxic, having several injurious effects ranging from stunted growth and leaf drop to the development of small twisted leaves in

ornamental and vegetable crops (Kelly, 1993). The use of Benlate in *in vitro* culture systems has also been reported to have phytotoxic effects on explant tissue (Kelly, 1993). In this study, high doses (1 $g.l^{-1}$) added to the culture medium resulted in death of the explants within two weeks. The explants were, however, apparently not affected by the fungicide wash solution which contained 1 $g.l^{-1}$ Benlate. Although fungal contamination was significantly reduced by adding Benlate and Amphotericin B to the wash solution and culture medium, this method of sterilisation is extremely expensive particularly if this species is to be mass propagated. An alternative means of sterilisation would need to be developed for this culture system to be economically feasible. Preliminary tests on an extract from the seed of another indigenous tree, *Trichilea dregeana*, have revealed that this extract (6 - 10% [v/v]), when applied to a fungal culture system, inhibited the growth of *Penicillium* spp., *Fusarium* spp. and *Cladosporum* spp. (Norris, pers. comm.). A similar study conducted by Norris has shown that extracts of *Allium sativum* also have strong anti-fungal properties. These extracts would prove extremely useful if effective in reducing or eliminating fungal growth from *Cryptocarya* cultures.

Another fungicide tested, Previcur N, had little effect in reducing fungal contamination in leaf and nodal segment cultures. However, as a precautionary measure, the fungicide was applied thrice-weekly (usually approximately 24 hours prior to harvesting) as a foliar spray. An interesting observation is that Previcur N has been found to induce extensive axillary budding in *Pinus* species (Watt *et al.*, 1995). It was found that, within one week, *in vivo* axillary bud induction occurred thus

providing a large amount of young and potentially uninfected tissue which could be used as explant material.

Bravo did not reduce fungal contamination in either nodal segment or leaf cultures (Table 4.7A and 4.7B). In fact, the fungicide, at the concentrations tested, appeared to promote fungal growth. When Bravo was used in combination with Benlate, fungal contamination was reduced by approximately 15 %.

In addition to the problem of fungal contamination, the extensive exudation of polyphenolics into the culture medium was addressed. Phenolics which became oxidised are released as a result of tissue injury during dissection and eventually the tissue becomes brown or black. The oxidation products are known to inhibit enzyme activity, darken culture media and tissues and are associated with death of the explant (Evans *et al.*, 1983). This impedes the establishment of primary cultures particularly in woody species. McCown and Newton (1981) have reported that dark incubation of the explants for a period of time reduces phenolic exudation into the medium. In this study, dark treatment had no effect on phenolic production. However, ascorbic and citric acids were found to be extremely successful (Figure 4.3) in eradicating contamination by these compounds.

4.4.2. Axillary bud proliferation

Vegetative propagation is an important component of *ex situ* conservation systems. Following the successful rapid multiplication of orchids (*Cymbidium* species) by shoot meristem culture (Morel, 1965), there has been great interest in the application

of tissue culture techniques as an alternative means of asexual propagation of economically important plants. Murashige (1974) developed the concept of developmental stages: Stage I: explant establishment; Stage II: multiplication of the propagule; and stage III: rooting and hardening for planting into soil. This suggested that a single medium was not sufficient for *in vitro* plant multiplication and regeneration. The greatest success using this technique has been achieved for herbaceous horticultural and crop species (Table 4.10). This success has been partly due to the weak apical dominance and strong root regenerating capacities of many herbaceous plants, and partially due to financial interest by the commercial sector. Compared with herbaceous plants, the micropropagation of woody species is limited. The greatest difficulty is experienced at Stages I and III. Problems are often encountered when the primary culture is established because of high concentrations of phenolics in woody tissues and also due to the difficulty of breaking the physiologically quiescent state of the axillary buds (Hu and Wang, 1985). Those authors also suggest that root induction (Stage III), is especially difficult when explants are obtained from mature trees.

As mentioned previously (Section 1.5), the success of any culture system depends on the use of the nutrient formulation and plant growth regulators capable of inducing the highest degree of axillary bud induction and multiplication. Several nutrient media have been formulated but the most commonly used for tree tissue culture are full- and half-strength MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1981), Heller (Heller, 1953) and B5 (Gamborg *et al.*, 1968) media. In this investigation, full-strength MS induced axillary budding on all explants whereas only 60% of explants

cultured on WPM grew buds. In addition, there was a high percentage (25%) of leaf and bud senescence on explants cultured on WPM when compared with those cultured on MS medium (0%).

Table 4.10: Some of the reported studies of successful herbaceous agronomic, ornamental and vegetable crops propagated via *in vitro* axillary bud proliferation.

Genus	Reference
Allium	Wang and Huang (1974)
Anigozanthos	McCown and Newton (1981)
Anthurium	Kunisaki (1980)
Arachis	Kartha *et al.* (1981)
Beta	Atanassov (1980)
Brassica	Kuo and Tsay (1977); Walkey *et al.* (1980)
Capsella	Walkey and Cooper (1976)
Capholatus	Adams *et al.* (1979)
Chrysanthemum	Earle and Langhans (1975)
Cicer	Kartha *et al.* (1981)
Fragaria	James (1979); Kartha *et al.* (1980)
Gerbera	Murashige (1974)
Gladiolus	Ziv *et al.* (1970)
Gloxinia	Murashige (1974)
Glycine	Evans (1981)
Gypsophila	Kusey *et al.* (1980)
Hosta	Papachatzi *et al.* (1980)
Lolium	Dale (1975, 1977, 1979, 1981)
Phaseolus	Kartha *et al.* (1981)
Phlox	Schnabdrauch and Sink (1979)
Saxifragra	Murashige (1974)
Stellaria	Walkey and Cooper (1976)
Stevia	Yang *et al.* (1981)
Vigna	Kartha *et al.* (1981)
Zea	Raman *et al.* (1980)

The merits of using axillary bud proliferation from meristem, shoot tip or bud cultures as a means of regeneration, is that the incipient shoot has already been differentiated *in vivo* (Hu and Wang, 1983). In contrast, in indirect organogenesis or embryogenesis the explant must undergo developmental changes which involve callus formation and subsequent differentiation into plantlets. In cultures making use of axillary shoot proliferation, cytokinin is used to overcome the apical dominance and to enhance branching of lateral buds from leaf axils (Hu and Wang, 1983). The effective concentration of exogenous cytokinin required to reverse apical dominance varies with culture systems and species. As mentioned previously, budding was induced on all explants cultured on MS medium supplemented with 1.0 $mg.l^{-1}$ BAP and 0.01 $mg.l^{-1}$ NAA and on other hormone combinations to a lesser extent (Table 4.8). The appropriate ratio of cytokinin:auxin was essential to the success of axillary bud induction and proliferation. A comparison between media containing BAP and NAA (Tables 4.8 and 4.9) shows that high ratios of the cytokinin:auxin induced greater axillary bud extension and proliferation than lower ratios. Koda and Okazaw (1980) have reported that although a small amount of cytokinin is synthesised by shoots *in vitro*, there may not be sufficient endogenous cytokinin to support growth and development. In this study, axillary bud proliferation was induced on the same medium found to be successful for bud induction (60% of explants). Benzylaminopurine was the most effective cytokinin for the stimulation of bud multiplication. Gibberellic acid did not have any effect on the buds although it has been found to be more effective than most cytokinin:auxin combinations (Evers *et al.*, 1988) (Table 4.8).

4.4.3. Callus formation

Callus cultures provide a means for producing plantlets (Chapter 1, Section 1.5.1): via either organogenesis or embryogenesis (Warren, 1991). In this study, callus formed on explants derived from the petiolar region of the leaf (Figure 4.2). Similar results have been obtained using *Eucalyptus* leaf explants (Gauntlett, pers. comm.)

4.4.4. Future research strategies

Fungal contamination posed a major problem to the establishment of leaf and nodal segment cultures of *C. latifolia*. A mixture of Benlate (0.1 $g.l^{-1}$) and Amphotericin B (0.0025 $g.l^{-1}$) reduced contamination to approximately 10 %. However, this method could prove extremely expensive especially for large-scale production and the preliminary work by Norris (pers. comm.) has shown that extracts from the seed of the indigenous tree, *Trichilea dregeana*, and from the cloves of *Allium sativum* do possess anti-fungal properties (see above). Testing of these extracts on *C. latifolia* tissue cultures is ongoing.

In the present investigation, several nutrient media and plant growth regulator combinations were tested to induce axillary bud induction and proliferation. Only one culture medium stimulated axillary bud proliferation (MS nutrients supplemented with 0.01 $g.l^{-1}$ biotin, 0.01 $g.l^{-1}$ calcium pantothenate, 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar, 0.01 $mg.l^{-1}$ NAA and 1.0 $mg.l^{-1}$ BAP). However, multiplied shoots were observed on only 60 % of explants using this treatment. Recently, Huetteman and Preece (1993) have reported that thidiazuron is a potent cytokinin and it has been shown to induce even greater axillary bud proliferation in woody species than other cytokinins (for example

BAP). Furthermore, very low concentrations (0.01 $mg.l^{-1}$) compared with other cytokinins are able to promote proliferation and shoot multiplication. Future research could therefore involve testing thidiazuron to improve proliferation rates in nodal segment cultures of this species.

Further research would involve testing and developing various media known to promote shoot elongation in woody species. In addition, rooting and hardening off protocols would need to be established.

Another problem in the *in vitro* culture of *C. latifolia* was that of regeneration via callus cultures either via organogenesis or embryogenesis. The callus formed (MS nutrients containing 30 $g.l^{-1}$ sucrose, 10 $g.l^{-1}$ agar, 3 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ kinetin) during this study was non-embryogenic and therefore regeneration could not be achieved. Huetteman and Preece (1993) have reported that thidazuron not only stimulates shoot multiplication at low concentrations, but also promotes the production of callus, adventitious shoots and somatic embryos at higher concentrations (0.001 $mg.l^{-1}$). Therefore, inclusion of this PGR, in place of other cytokinins, should be tested.

CONCLUDING REMARKS

The technique of micropropagation is widely used in agriculture, horticulture and conservation for the mass propagation, selection and genetic transformation of selected genotypes. This study was aimed at the development of micropropagation protocols for three indigenous species commonly used in traditional medicines, *Bowiea volubilis*, *Haworthia limifolia* and *Cryptocarya latifolia*.

Bowiea volubilis and *H. limifolia* were successfully propagated using two different routes of differentiation. *Bowiea volubilis* was propagated via direct organogenesis producing, on average, four plantlets per explant. In contrast, *H. limifolia* was propagated via somatic embryogenesis with over twenty plantlets being produced per explant. Preliminary studies on *Cryptocarya latifolia* resulted in the establishment of a sterilisation protocol as well as a medium for the induction of axillary buds and the multiplication of shoots. However, for this species protocols must still be developed for shoot elongation and rooting before the resulting plantlets can be acclimated to ambient conditions.

A major problem encountered in the propagation of *H. limifolia* was that of vitrification. This set-back was overcome by incubating vitrified plantlets at lower relative humidity. This strategy reversed the vitrified condition and plantlets showed normal morphological and anatomical characteristics within 4 - 6 weeks of silica gel being introduced. These healthy plantlets were readily hardened-off, in contrast to the

vitrified plantlets which could not be successfully acclimated to greenhouse conditions.

A second problem - one not directly related to the micropropagation protocols - involves the commercial application of these techniques. Plants generated *in vitro* may not be readily accepted by the communities for use in traditional medicines. These plants are not considered to have the same potency as plants growing in the wild (Iwu pers. comm.; Mkhize, pers. comm.). Plants growing in natural habitats are perceived to have distinct relationships with other elements within the ecosystem. Factors such as the communication between two or more plant species, nutrient recycling within the ecosystem, the interaction of plant and soil microbes and even material dropping from the canopy to ground level is believed to influence the effectiveness of medicinal species. The problem of acceptance may well be overcome by introducing plants regenerated *in vitro* into conservancies or ethnobotanical reserves and harvesting them on a manageable scale so that this resource is sustainable. Such plants may be more acceptable than those growing in pots.

The irretrievable loss of naturally-occurring germplasm due to the over-utilisation of medicinal plants is cause for concern. The introduction of large numbers of plants generated *in vitro* into reserves not only makes these plants more acceptable - it also allows for the conservation of germplasm and thus the maintenance of biodiversity. An alternative method for the preservation of germplasm is the cryopreservation of plant material. The material (most significantly embryos and shoot tips) developed *in vitro* should be ideally suited to cryopreservation techniques, and the development of

protocols for the cryopreservation of these tissues will ensure a permanent supply of important species.

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