In vitro conservation of endangered *Dierama* species

BY

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This thesis is dedicated to my late father, Michael (Bra Mike) Madubanya

DECLARATION

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

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DECEMBER 2004

We certify that the above statement is correct

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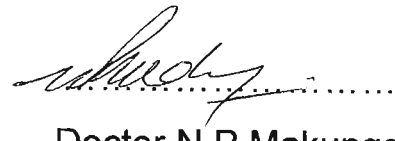

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ABSTRACT

Dierama belongs to the Iridaceae family. This genus can be distinguished from other members in the family by its long arching stems that carry the weight of the bell-shaped flowers. The species are only found in moist grasslands and these habitats have been greatly exploited by forestry and agriculture. Vegetative propagation via corms has proved to be a slow process while seed viability is greatly affected by beetle predation. A number of species endemic to the KwaZulu-Natal Midlands are considered endangered. Tissue culture was used as a conservation tool to propagate plants. In future this can be used in the commercialization of endangered species.

Dierama corms were decontaminated using 70 % ethanol and 3.5 % NaOCl (full strength JIK). There was little success even after using hot water at 50 and 55 °C to eliminate the endogenous contamination. *D. luteoalbidum* and *D. erectum* seeds were successfully decontaminated with 70 % ethanol and 3.5 % NaOCl (JIK). The seeds were germinated *in vitro* on MS medium with or without 0.5 mgL^{-1} BA. A better germination response was achieved on a medium devoid of BA. *D. luteoalbidum* seeds had the lowest germination response owing to the age of the seeds.

Hypocotyl explants formed multiple shoots on MS medium supplemented with 0.5 mgL^{-1} BA. Increasing the BA concentration and adding 1.0 mgL^{-1} NAA led to the formation of callus. The shoots were transferred to liquid MS medium supplemented with 0.5 mgL^{-1} BA for mass propagation. After six to eight weeks the shoots were reduced to meristemoid clusters that formed secondary shoots on solidified MS medium after three to four weeks. The shoots formed roots on solidified MS medium with 6-8 % sucrose after three months. There was no significant promotion of rooting when 5 gL^{-1} activated charcoal was added to the growth medium. Shoots left undisturbed formed corms *in vitro* on 6-8 % sucrose supplemented MS medium after six months. Other shoots transferred to MS medium supplemented with 1-10 mgL^{-1} paclobutrazol formed corms and stunted

roots after three months. The rooted shoots were transferred to the mist house for three to four weeks for hardening-off where they showed 80-100 % survival. In the greenhouse, more plantlets survived in the peat: compost: bark (1:1:1) v/v mixture than in the soil: sand: vermiculite (1:1:1) v/v potting mixture. Corms were formed after six months and the Kelpak-treated plantlets formed larger corms than the tap-watered plants.

The genetic fidelity of the micropropagated plants derived from meristemoid clusters was determined using RAPD. DNA was extracted from *in vitro* leaves using a modified CTAB DNA extraction method. Extracting DNA from plantlets in the culture jars yielded more DNA than extracting DNA from leaves stored at -70 °C. From the 24 primers used in the primer screening process, only six were selected for further analyses. Three out of the six primers namely: OPB-11, OPB-18 and OPC-01 yielded little genetic variation in the micropropagated plants. This suggested that meristemoids can be used in the clonal propagation of *Dierama* species for conservation purposes.

D. luteoalbidum seeds had a poor germination response despite using dormancy-breaking techniques. This highlighted the need to study the effect of storage on seed viability. *D. igneum* seeds had an average moisture content of 15 % meaning they could be classified as orthodox. These seeds were stored for a period of 6 months at temperatures ranging from 0 to 30 °C without losing viability. Seeds germinated at 25 °C and took only 10 days to reach 100 % germination. There was no germination recorded at 10 and 40 °C. The seeds only germinated once the temperature was shifted to 25 °C.

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LIST OF ABBREVIATIONS USED

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
A_{320}	Absorbance at 320 nm
A	Adenine
Tris	2-Amino-2(Hydroxymethyl)-Propane-1,3-Diol
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
±	Approximately
bp	Base Pairs
BA	6-Benzyladenine
cm	Centimetre
cv	Cultivar
C	Cytosine
°C	Degrees Centigrade
DNA	Deoxyribose Nucleic Acid
DNTP	Deoxynucleoside Triphosphate
2,4-D	2,4-Dichlorophenoxyacetic Acid
DAF	DNA Amplified Fragment
EDTA	Ethylene Diamine Tetra Acetate
FW	Fresh Weight
g	Grams
gL^{-1}	Grams per Litre
g	Gravitational Acceleration (9.806 $m.sec^{-1}$)
GI	Growth Index
G	Guanine
CTAB	Hexadecyl-trimethylammonium Bromide
HPLC	High Power Liquid Chromatography
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
IPA	N^6-(isopentyl)-Adenine
kb	Kilobase Pair

LSD	Least Significant Difference
<	Less than
$HgCl_2$	Mercuric Chloride
µg	Microgram
$\mu g\mu l^{-1}$	Microgram per Microlitre
µl	Microlitres
$\mu mol\ m^{-2}\ s^{-1}$	Micromole per Square Metre per Second
mg	Milligrams
mgL^{-1}	Milligram per Litre
ml	Millilitres
mm	Millimetres
mM	Millimolar
Min	Minutes
MS	MURASHIGE and SKOOG (1962) nutrient medium
ng	Nanogram
nm	Nanometre
NAA	α-Naphthaleneacetic Acid
n	Number
OP (A-C)	Operon Primer Kit (A-C)
%	Percent
PCR	Polymerase Chain Reaction
PVPP	Polyvinylpolypyrrolidone
P	Probability
RAPD	Random Amplified Polymorphic DNA
®	Registered
RH	Relative Humidity
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per Minute
SRFA	Selective Restriction Fragment Amplification
SSR	Simple Sequence Repeats
NaCl	Sodium Chloride
NaOCl	Sodium Hypochloride
sp.	Species

S. E.	Standard Error
T	Thymine
X	Times
™	Trade Mark
TTC	2,3,5-Triphenyl Tetrazolium Chloride
TAE	Tris Acetic Acid
TE	Tris EDTA
Tris-Cl	Tris Hydrochloride
UV	Ultra-violet
VNTR	Variable Number of Tandem Repeats
v/v	Volume per Volume
H_2O	Water
w/v	Weight per Volume

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CHAPTER 1: INTRODUCTION

The genus *Dierama* is of horticultural importance. Their pendulous, bell-shaped flowers are their most striking feature. The flower colour ranges from white to pink to dark red. These plants have a narrow distribution in the wild, limited to only moist grasslands. These habitats have been significantly reduced by forestry and agriculture. *Dierama* species can easily be propagated from seeds and corms. This, however, is a very slow process. Furthermore, survival in the wild is affected by beetles. *In* vitro propagation techniques have proved over the years to be helpful in mass propagation of endangered plants. To check for the genetic stability of the plantlets derived from tissue culture, DNA fingerprinting techniques like RAPD can be used. There are reports of *in situ* conservation of endangered species like *Dierama*. However, for *ex situ* conservation of *Dierama* which involves the storage of germplasm, no information is available. Seeds are by far the most convenient plant parts for storage and are well adapted for this. The horticultural potential and rarity of *Dierama* species in the wild provided the stimulus for this work, namely to micropropagate these threatened plants for conservation purposes.

1.1 Morphology

a) Iridaceae

The Iridaceae is a fairly large family within the monocotyledons. It consists of 65 genera and about 1800 species (GOLDBLATT, 1971). The family is divided into two main groups: 1) rhizomatous; and, 2) bulbous species (JEPPE, 1999). The family is distributed worldwide but the region of greatest concentration is southern Africa. In southern Africa alone there are about 45 genera and 900 species (GOLDBLATT, 1971). The family is homogenous and is relatively easy to recognize among the petaloid monocotyledons; the flowers have only three stamens and the ovary is inferior (HILLIARD and BURTT, 1991).

According to GOLDBLATT (1971) the family is further divided into subfamilies Irideae and Ixideae; more recently referred to as Iridoideae and Ixioideae (GOLDBLATT, 1990). The members in the different subfamilies are summarized in Table 1 below:

Table 1: The classification of members of the Iridaceae family (GOLDBLATT, 1971 and 1990)

Ixioideae	Iridoideae
Watsoniineae	Dietes
Freesiineae	Moraea
Lapeirousiineae	Gynandrisis
Hesperanthineae	Homeria
Crocineae	Galaxia
Gladiolineae	Hexaglottis
Ixiineae	Ferraria
Tritoniineae	Aristea
Babianineae	Sisyrinchium
Exohebineae	
Sparaxis	
Schizostylis	
Syringodea	
Geissorhiza	
Pillansia	
Dierama	

b) *Dierama*

The genus *Dierama* is most commonly known as Hairbells in Africa and Wandflowers when cultivated in Britain (HILLIARD and BURTT, 1991). In Ixioideae, the sub-family to which *Dierama* belongs, the flowers are subsessile, arranged in indeterminate spikes and seldom fugitive; the nectaries lie between the septa of the ovary (HILLIARD and BURTT, 1991). The name *Dierama* comes from an ancient Greek word meaning a funnel, which refers to the shape of the perianth, the upper conspicuous bell-shaped cone narrowing sharply into a tubular shaft. The genus *Dierama* generally shows very little structural variation among the species, the key features being evergreen leaves, corms and flowering stalks (HILLIARD and BURTT, 1991).

All Dieramas have corms and these are surrounded by a sheath of tough fibres derived from the bases of dead leaves. In nature the corm is often deep-seated in the soil and is difficult to dig up undamaged. Each year a new corm is formed by the accretion of food reserves near the base of the flowering stem. Thus the new corm comes to sit atop the old and in due course, because the old corms are slow to decay, a chain of corms one above the other is formed. The corm attains its deep position in the ground, as do many other corms and bulbs, by the development of strong fleshy contractile roots. These penetrate deeply into the soil so that they are strongly anchored; then they begin to lose moisture and contract the upper part, as can be seen from numerous transverse wrinkles that develop (HILLIARD and BURTT, 1991).

If the fibres of the corm are stripped from the outside of a corm, it will be found to be a rather depressed globose structure marked by two to four circular lines. These lines are the scars of the leaves of the old flowering stem, each line representing a node (the insertion of a leaf), and it is easy to count how many nodes and internodes (the leafless sections of stem between the nodes) took part in the formation of the corm. Since the enlargement of the basal internodes of the flowering stem forms the new corm, its true apex is the scar of that stem; it does not have an apical bud of its own. When the new corm itself produces a

flowering stem, this may seem to arise at the apex of the corm, but it actually grows from the axil of a leaf on the previous year's stem at the top of the section where the corm was formed. Thus, the structure of the whole plant body of *Dierama* is a series of segments, each consisting of a shoot with leaves at its base and terminal inflorescence and a new corm forming in its basal region. The new segment starts with the new inflorescence arising from an axillary bud of a leaf at the base of the old stem now on the new corm (HILLIARD and BURTT, 1991).

1.2 Horticultural value

Dierama is a plant of unusual grace and beauty and it must have been regarded as an exciting find by early botanists. It was later introduced to gardeners in Europe. *Dierama igneum*, the plant originally introduced as *Sparaxis pendula* before it was renamed, was grown in gardens in London. It was K. Koch, a Professor of Botany, who found *Dierama pendulum* in the Botanic Garden in Berlin and determined that it was different to *Sparaxis pendula*. *D. pulcherrimum* was found thriving in west coast gardens in Scotland. There were a number of cultivars that were developed in Europe. The Slieve Donard Nursery in Ireland obtained a number of *Dierama* species, with the first species being *D. pulcherrimum* var. *album* in 1921, which was known since 1898. The other cultivars that were developed were 'Heron' (1923), 'Kingfisher' (1924), 'Windhover' (1928), 'Skylark' (1934) and 'Falcon' (1938) (HILLIARD and BURTT, 1991).

D. pendulum (pink) and *D. pulcherrimum* (purple) were also found growing outside Britain; *D. pulcherrimum* in California gardens (BAILEY, 1902; cited by HILLIARD and BURTT, 1991), where it had been growing for many years. They were also found in France (FOURNIER, 1951; cited by HILLIARD and BURTT, 1991), Australia (COOKE, 1986; cited by HILLIARD and BURTT, 1991), Tasmania and New Zealand (HEALY and EDGAR, 1980; cited by HILLIARD and BURTT, 1991). These species were again described in *Hortus Third* (BAILEY and BAILEY, 1976; cited by HILLIARD and BURTT, 1991) and in addition to

D. intermedium, a hybrid of the two former species (HILLIARD and BURTT, 1991).

Plate 1: *D. luteoalbidum* inflorescence indicating the long arching stem and bell-shaped flowers

1.3 Medicinal and other uses

The long tough leaves of *Dierama* can be used as cordage. *D. robustrum* is used as a source of fibre in the Cathedral Peak area of the Drakensberg. The Zulu people call it "iThembu" and regard *Dierama* leaves as the strongest fibre source available locally (KILLICK, 1963; cited by HIILIARD and BURTT, 1991). The use of *Dierama* for cordage was widespread in earlier times (HILLIARD and BURTT, 1991).

The corms of *Dierama* are not edible but they are used medicinally. In Lesotho, where it is called "Lethepu", *D. pendulum* is boiled and the product is used as an enema (HILLIARD and BURTT, 1991). In the former south-eastern Transvaal, *D. insigne* corms are dug in quantity in winter and are used for remedial purposes. Again on the farm Mooihoek near Piet Retief, *D. tyrium* is harvested in large quantities and used as a stomach medicine (HILLIARD and BURTT, 1991).

1.4 Distribution

Dierama may be found ranging from Knysna in the southern Cape, northwards through southern and eastern Africa. The genus comprises 44 species (HILLIARD and BURTT, 1991). The number of species recorded in each African region is as follows (Table 2):

Table 2: The number of *Dierama* species recorded in each African region (HILLIARD and BURTT, 1991)

Area	Number of species
Ethiopia	1
East tropical Africa (Uganda, Kenya, Tanzania)	4
Democratic Republic of Congo (formerly Zaire)	1
South tropical Africa (Malawi, Zambia, Zimbabwe, Mozambique)	7
Limpopo (formerly Transvaal)	14
Swaziland	8
Free State	6
Lesotho	5
KwaZulu-Natal	26
Eastern Cape	13
Western Cape	11

The main centre of diversity of this genus is south of Limpopo (Table 2) with KwaZulu-Natal harbouring the greatest number of species. This is linked with the fact that this province provides suitable habitats for *Dierama* from sea level to 3000 m. Throughout its distribution *Dierama* shows very slight ecological amplitude, the amplitude always being essentially moist grassland. In the tropics it is montane or submontane. In KwaZulu-Natal and further south *Dierama* is found down to sea level. This restriction of a habitat is accompanied by marked structural uniformity (HILLIARD and BURTT, 1991).

The absence of *Dierama* from the winter-rainfall area of the Cape sheds light on the presence and diversification of other related genera. In contrast to *Dierama*, the other species have developed a life cycle attuned to a short season favourable to growth, and a floral diversity adapted to a range of pollinators. The geomorphology of the Cape Mountains is rugged and the soils are essentially poor and acid compared with the richer soils of the valleys and coastal plains; there are also marked differences in local climates within the general area of

winter rainfall (BOND and GOLDBLATT, 1984; cited by HILLIARD and BURTT, 1991).

KwaZulu-Natal not only has the greatest number of species but it also has the widest range of flower colour. Only in KwaZulu-Natal are pale yellow or cream-coloured perianths such as those of *D. luteoalbidum, D. pallidum, D. pumilum* and *D. sertum* to be found, while at the other end of the spectrum the flowers of *D. dubium* and (in the adjacent Transkei) *D. atrum* are so dark a red as to justify the epithet *atrum* (black). *D. argyreum* and perhaps the dubious *D. elatum* from Swaziland are the only wild species with white flowers, and in *D. argyreum* they are often tinged pink or mauve (HILLIARD and BURTT, 1991).

It is not well known whether the diversity in flower colour is attributed to pollinators since very little is known about the pollination of *Dierama*. These plants usually set seeds freely and in cultivation this is true of isolated plants, so that, in some species at least, there is clearly no self-incompatibility. The flowers are certainly visited by bees, both in the garden and in the wild, and these are probably the normal pollinating agents. It was mentioned that the bells are the haunt of a crab-spider, delicately coloured mauve like its background so that it is almost invisible; indeed it is the unfortunate captured bee that draws attention to the spider (HILLIARD and BURTT, 1991).

1.5 Conservation

There are about 11 endemic *Dierama* species recorded to date in KwaZulu-Natal. Five out of the eleven species namely, *D. erectum, D. luteoalbidum, D. nixonianum, D. pallidum* and *D. pumilum* are regarded as vulnerable in their natural habitats. These endemic species are vulnerable because they are rare; showing a narrow distribution and a very low abundance in their habitats. The vulnerability can also be attributed to grassland transformation by agriculture and forestry (SCOTT-SHAW, 1999).

Owing to the conservation status of the species, a number of future needs have been identified. There is an urgent need to conserve the endemic species and so far all of the species found in the KwaZulu-Natal Mistbelt Grassland are legally protected in their natural habitats. Furthermore, the plants need to be propagated to provide an *ex situ* reserve of propagules. There is also a need to monitor populations to improve habitat management. For *D. erectum* there is a need to investigate the optimal fire management regimes (SCOTT-SHAW, 1999).

1.6 Storage of germplasm

Germplasm is a collection of genetic material from which a whole organism can be recovered. In the case of plants, germplasm may be seeds, shoot cultures or regenerable cell cultures (STAFFORD and WARREN, 1991). When storing germplasm, the following need to be considered: a) lines that do not form viable seeds; b) lines that produce recalcitrant seeds; c) seed sizes that are so large that storage of sufficient numbers would require large amounts of space; d) lines that possess long juvenile seeds; and, e) heterozygous clones that arise as a result of continued crossing (TOWILL, 1988).

There are many genetic issues related to the collection and maintenance of species. Collection is, in principle, the same among different crops, assuming something is known of the species' breeding system and population structure. There are, however, many practical considerations that differ in obtaining adequate samples of individuals and populations (TOWILL, 1988). Once the materials have been collected, the genetic composition must be maintained by the best preservation and propagation procedures available. The balance between practical and optimal is based on costs and time available (TOWILL, 1988). Accurate characterization of the clone is required to answer questions related to genetic stability. Characterization and evaluation should include data on morphology and phenology, agronomic or horticultural performance, physiological aspects and electrophoretic analyses. Both nuclear and organelle genomes (chloroplast or mitochondria) analyses may be useful in characterizing lines (TOWILL, 1988).

1.7 Conservation through propagation

There are two propagation methods: a) conventional and, b) *in vitro* propagation.

a) Conventional propagation

Dierama species produce corms, which are swollen stem bases in which food reserves are stored. These corms have three to four nodes and a number of axillary buds (GEORGE, 1993). From one corm, several small daughter corms usually arise at the base towards the end of the current year's growth. The plants are evergreen as they never go into a state of complete dormancy, and therefore nurserymen do not sell resting corms, as they do of *Crocus* and *Gladiolus. Dierama,* being plants from the summer-rainfall areas of Africa, require plenty of water in the growing season. Although they like the sun, they are not plants for a hot, dry border. New plants can be initiated from these small cormlets (GEORGE, 1996). This natural vegetative multiplication is slow and if interest is to commercially propagate the plant, then this method would be inappropriate.

Alternatively, *Dierama* may easily be grown from seed (HILLIARD and BURTT, 1991). The advantages of propagation using seeds are: a) seeds are often produced in large numbers so that plants regenerated from them are individually inexpensive; b) in general, seeds may usually be stored for long periods without the loss of viability; c) seeds are easily distributed; and, d) plants grown from seeds are without most of the pests and diseases which may have afflicted their parents (GEORGE, 1993). The disadvantages, however, are that the seeds of many plants produce genetically different plants and to obtain seeds which give uniform offspring is either difficult or impossible practically (GEORGE, 1993). If it is desirable to obtain true-to-type offspring, that is, clones or populations that are identical to the mother plant, corms are preferred. The other disadvantage is that the *Dierama* seeds collected from the wild or from gardens in Africa, may be attacked by the grub of a bruchid beetle (*Urodon lilii*). The female beetle lays its eggs on the outside of the young ovary and the grub burrows through the wall

into an ovule. Whatever happens inside the ovule, the development of the outer layers into a normal looking brown and shining seed coat seems not to be impeded. When ripe seeds are collected some of them may feel slightly soft and when opened will be found to contain a neatly rolled beetle-grub. Good beetle-free seeds may be sown in pots or directly into the ground where the plants are able to grow. The germination rate of *Dierama* seeds is very high, so the seeds should be well spaced. *Urodon lilii* is not restricted to the seeds of *Dierama*; it is equally common in some other South African Iridaceae such as *Moraea, Dietes* and *Watsonia* (HILLIARD and BURTT, 1991).

It is therefore necessary to find other ways of propagation that will counteract the problems encountered with conventional propagation.

b) *In vitro* propagation

In vitro techniques that have been developed are usually an extension of those already developed for conventional propagation.

i) Advantages

The advantages of *in vitro* techniques over traditional techniques are the following:

1) Cultures are started with very small pieces of plants (explants), and thereafter small shoots or embryos are propagated (hence the term micropropagation). Only a small amount of space is required to maintain plants or to greatly increase their number;
2) Propagation is ideally carried out in aseptic conditions, free of pathogens. Once cultures have been started there should be no loss through disease, and the plantlets finally produced should be free of bacteria, fungi and other micro-organisms;
3) Methods are available to free plants from virus disease. Provided these techniques are employed, certified virus-free plants can be produced in large numbers;

4) A more flexible adjustment of factors influencing vegetative regeneration is possible such as nutrients and light, temperature and plant growth regulator levels. The rate of propagation is greater than in macro-propagation and many more plants can be produced in a given time. This may enable newly selected varieties to be made available quickly and widely, and numerous plants to be produced in a short while. The technique is very suitable when high volume production is essential;
5) It may be possible to produce clones of some kinds of plants that are otherwise slow and difficult (or even impossible) to propagate vegetatively;
6) Plants may acquire a new temporary characteristic through micropropagation which makes them more desirable to the grower than conventionally raised stock;
7) Production can be continued all year round and is independent of seasonal changes;
8) Vegetatively-reproduced material can often be stored over long periods;
9) Less energy and space are required for propagation purposes and for the maintenance of stock plants; and,
10) Plant material needs little attention between sub-cultures and there is no labour or materials required for watering, weeding or spraying. Micropropagation is most advantageous when it costs less than traditional methods of multiplication. If this is not the case there must be some other important reason to make it worthwhile (GEORGE, 1996).

ii) Disadvantages

The chief disadvantages of *in vitro* methods are that advanced skills are required for their successful operation; a specialized and expensive production facility is needed; fairly specific methods may be necessary to obtain optimum results from each species and variety; and, because present methods are labour intensive, the cost of propagules is usually relatively high. Other disadvantages of using *in vitro* techniques are:

1) Although they may be produced in large numbers, the plantlets obtained are initially small and sometimes have undesirable characteristics;
2) In order to survive *in vitro*, explants and cultures have to be grown on a medium containing sucrose or some other carbon source. The plants derived from culture are not initially able to produce their own requirement of organic matter by photosynthesis and have to undergo a transitional period before they are capable of independent growth;
3) As they are raised within glass or plastic vessels in a high relative humidity, and are not usually photosynthetically self sufficient, the young plantlets are more susceptible to water loss in an external environment. They may therefore have to be hardened in an atmosphere of slowly decreasing humidity and increased light; and,
4) The chances of producing genetically aberrant plants may be increased (GEORGE, 1996).

iii) Outline of the micropropagation steps

Stage 0: Mother plant selection and preparation

Stage 0 is the selection and preparation of mother plants. The main objective of this stage is to select and grow mother plants that are healthy and true-to-type. The use of low humidity (75 %) and no overhead watering often reduces bacterial, fungal and viral contamination. In addition, mother plants can be exposed to specific temperatures or light treatments that reduce infections (KIM and DE HERTOGH, 1997).

Stage 1: Establishing an aseptic culture

Stage 1 is the establishment of the aseptic culture. Juvenile explants have been shown to be most responsive. The objective of this stage is to decontaminate the explants. This is achieved by using decontaminants such as Ethanol, Mercuric chloride ($HgCl_2$), NaOCl (JIK®), fungicides (e.g. Benlate™ and Sporekill™) and sometimes antibiotics. Browning caused by oxidation can be prevented by anti-

oxidants such as ascorbic acid (DE BERGH and READ, 1991; KIM and DE HERTOGH, 1997). Activated charcoal can also be used to prevent browning. It adsorbs certain inhibitory substances in culture, such as phenolic compounds (PAN and VAN STADEN, 1998).

Stage 2: The production of suitable propagules

Stage 2 is the multiplication of propagules. Multiplication of shoots and bulblets obtained from explants can be achieved by: 1) enhancement of axillary bud growth; 2) proliferation of adventitious shoots; 3) induction of adventitious buds from callus; and, 4) plantlet regeneration by somatic embryogenesis. Generally supplementation of the culture medium with cytokinins or plant growth retardants maximizes shoot proliferation. Cytokinins may also have negative effects. For example, they may decrease the degree of rooting or bulblet formation and also may decrease the survival of plantlets when transferred from *in vitro* to *ex vitro* conditions. The medium in stage 2 is enriched with substances that enhance organogenesis; especially shoot formation and in the case of ornamental geophytes, bulblets (MURASHIGE, 1974; KIM and DE HERTOGH, 1997).

Stage 3: Preparation for growth in the natural environment

Stage 3 is the obtaining, hardening and bulbing of plantlets. The objective of this stage is to obtain plantlets and bulblets from shoots for transfer into the soil. ZIV (1979) reported that plantlets are directly transplanted to reduce losses of small bulblets and to prevent dormancy. Rooting is achieved in three ways: 1) adding activated charcoal to the culture medium; 2) increasing the auxin to cytokinin ratio; and, 3) using half strength salts and sucrose (ZIV, 1979; KIM and DE HERTOGH, 1997).

c) *In vitro* propagation of Iridaceae species

Some members of the Iridaceae that have been successfully propagated *in vitro* include *Gladiolus, Crocus* and *Freesia* (GEORGE, 1996). The table below shows a summary of the *in vitro* work achieved for Ixioideae species (Table 3).

Table 3: Summary of *in vitro* studies conducted in the Ixioideae sub-family

Species	Explant	Type of culture	Growth response	Reference
Babiana angustifolia (Salisb. Ex Lewis) Gold tel.	Seedling hypocotyl	Direct	Multiple shoots, callus and root formation	McALISTER *et al.* (1998)
Babiana disticha Ker.	Seedling hypocotyl	Direct	Multiple shoots, callus and root formation	McALISTER *et al.* (1998)
Babiana patersoniae L. Bolus	Seedling hypocotyl	Direct	Multiple shoots, callus and root formation	McALISTER *et al.* (1998)
Babiana rubrocyanea (Jacq.) Ker.	Seedling hypocotyl	Direct	Multiple shoots, callus and root formation	McALISTER *et al.* (1998)
Babiana stricta (Ait.) Ker. Var. *regia* Lewis	Seedling hypocotyl	Direct	Multiple shoots, callus and root formation	McALISTER *et al.* (1998)
Babiana villosa (Ait.) Ker.	Seedling hypocotyl	Direct	Multiple shoots, callus and root formation	McALISTER *et al.* (1998)
Gladiolus grandiflorus cv. 'Jackson Ville' and 'Peter Pears'	Terminal and lateral buds of corms	Shoot	Increased number of shoots/callus explant	EL-GENDY *et al.* (2001a)

Table 3: continued

Species	Explant	Type of culture	Growth response	Reference
Gladiolus grandiflorus cv. 'Jackson Ville' and 'Peter Pears'	Terminal and lateral buds of corms	Direct/callus	*In vitro* cormel production	EL-GENDY *et al.* (2001b)
Gladiolus cv. 'Friendship'	Corm axillary buds	Direct	Multiple shoot formation and corm formation	HUSSAIN *et al.* (1997)
Gladiolus sp.	Root	Callus	Plantlet regeneration and corm formation	MOHAMED (2000)

Table 3: Summary of *in vitro* studies conducted in the Ixioideae sub-family (GEORGE, 1996)

Species	Explant	Type of culture	Growth response	Reference
Crocus sativus L. (Saffron)	Ovaries at pre- or post- anthesis	Fruit	Parthenocarpic fruits	CHICHIRICCO and CAIOLA (1987)
Crocus sativus L. (Saffron crocus)	Apical and lateral buds	Direct	*In vitro* formation of corms	MILYAEVA *et al.* (1995)
Freesia x *hybrida* [19 cvs.]	Young flower ovaries	Callus	Adventitious shooting and rooting *in vitro*	BACH (1987)
Freesia x *hybrida* L.H. Bail.	Young flower buds	Callus	Adventitious shoot formation	BAJAJ and PIERIK (1974)
Freesia x *hybrida*	Corms and apical meristems	Callus	Adventitious shoot formation and plantlets rooted	PETRU *et al.* (1976)
Freesia x *hybrida*	Meristem tips	Meristem	Virus free plants	BRANTS (1968)
Freesia x *hybrida*	Flower pedicels	Callus	Shoot regeneration	STIMART and ASCHER (1978b; 1982)

Table 3: continued

Species	Explant	Type of culture	Growth response	Reference
Freesia x *hybrida*	Corm tissue	Direct and callus	Adventitious shoot formation and plant regeneration	HUSSEY (1975b)
Freesia x hybrida	Pretreated soft stem sections	Direct and shoot	Adventitious and axillary shoot formation	HUSSEY (1977a)
Freesia x hybrida	Meristem tips	Meristem	Virus free plants	HOLLINGS (1965)
Freesia x *hybrida*	Young flower buds	Direct	Shoot bud formation and direct rooting	PIERIK and STEEGMANS (1975a)
Freesia x *hybrida*	Flower buds	Direct	Adventitious shoot formation and proliferation	PIERIK and STEEGMANS (1976b)
Freesia x *hybrida*	Aerial corm sections	Direct	Adventitious shoots with roots	READ and GAVINLERTVATANA (1976b)
Gladiolus cv. '*Yamit*' [miniature hybrid]	Infected apical meristems	Meristem	Virus free and infected plantlet regeneration	LILIEN-KIPNIS *et al.* (1992)

Table 3: continued

Species	Explant	Type of culture	Growth response	Reference
Gladiolus [6 cvs. Miniature hybrids]	Terminal and apical buds	Shoot	Multiple shooting and either rooting or dormant bulbils	LILIEN-KIPNIS and KOCHBA (1987)
Gladiolus cv. 'Eurovision'	Apical buds	Direct and shoot	Multiple bud formation then cormlet formation	ZIV (1989)
Gladiolus dalenii and *G. tristis*	Corm section	Direct	Shoot formation and later corm production	DE BRUYN and FERREIRA (1992)
Gladiolus grandiflorus	Cormel and flower stalk segments	Callus/direct	Entire plants regenerated	BAJAJ *et al.* (1983)
Gladiolus hortulans	Young inflorescence stalk	Callus/direct	Adventitious shoot and cormel formation	ZIV *et al.* (1970)
Gladiolus hybrid cv. 'Kinneret'	Terminal and lateral buds	Shoot and direct	Shoot proliferation followed by corm formation	STEINITZ *et al.* (1991)
Gladiolus sp.	Cormel stem tips	Callus	Shoot, root, cormel and embryogenesis	SIMONSON and HILDEBRANDT (1971)

Table 3: continued

Species	Explant	Type of culture	Growth response	Reference
Gladiolus sp.	Corm tissue and inflorescence stems	Callus and direct	Direct adventitious shoots	HUSSEY (1975b)
Gladiolus sp.	Corm axillary buds	Shoot	Axillary shoot proliferation	HUSSEY (1976c; 1977a,b)
Ixia flexuosa	Corms	Callus	Bud proliferation and rooting	MEYER and VAN STADEN (1988)
Schizostylis coccinea Backh. and Harv. (Kaffir lily)	Inflorescence stem	Direct	Direct adventitious shoot formation	HUSSEY (1975b; 1976c)
Schizostylis coccinea	*In vitro* shoots	Shoot	Axillary shoot proliferation	HUSSEY (1975b; 1976c)
Sparaxis bicolor	Corm and inflorescence stem	Direct	Direct adventitious shoot formation and proliferation	HUSSEY (1975b)

d) *In vitro* propagation of *Dierama* species

Very limited work has been reported on the successful propagation of *Dierama* species. PAGE and VAN STADEN (1985) reported on the *in vitro* propagation of *Dierama latifolium* with the aim of eventually obtaining somatic hybrids, as outlined below.

i) Decontamination of explants

The material was propagated using corm cultures. The newly developed corms were scrubbed under running tap water and then immersed in a solution of 100 % (v/v) alcohol for 5 minutes. Under aseptic conditions, the material was disinfected further by immersing it in a 0.1 % (w/v) mercuric chloride ($HgCl_2$) solution for 20 minutes and then rinsed twice with sterile distilled water. The basic nutrient medium (BM) consisted of the MS salt mixture with 30 gL^{-1} sucrose, 100 mgL^{-1} myo-inositol, 10 gL^{-1} Difco Bacto agar and adjusted to pH 6.0 (PAGE and VAN STADEN, 1985).

ii) Shoot proliferation

Shoots were induced from corm explants when grown on solidified MS medium supplemented with 30 gL^{-1} sucrose, 100 mgL^{-1} myo-inositol, and 0 or 0.5 mgL^{-1} NAA. Shoot proliferation was not improved following the addition of BA. Multiple shoots were induced by transferring those produced *in vitro*, to a modified MS medium supplemented with 0.5 or 1.0 mgL^{-1} BA. Subculturing single excised shoots, 5-10 mm in height, to either a hormone-free basal medium or a BA medium supplemented with 0.5 or 1.0 mgL^{-1} NAA induced rooting of these shoots (PAGE and VAN STADEN, 1985).

1.8 Germplasm: assessing the genetic diversity

After germplasm has been collected and stored the step that follows is assessment of the genetic diversity. In the case of *in vitro* produced plantlets that

are derived from callus, it is essential to extract DNA from those plantlets and analyse these at the genetic level. Plants derived from callus are thought to be genetically different. Callus induction can occur via the division of somatic cell types and it is likely that cultures arising from such complex mixtures of cells are themselves mixed populations from an early stage (STAFFORD, 1991).

A DNA fingerprint is a display of a set of DNA fragments from a specific DNA sample. When DNA fingerprints of related samples are compared, common bands as well as different bands will be observed. When these differences are observed in an otherwise identical fingerprint, such differences are referred to as DNA polymorphisms. DNA fingerprinting can therefore be used to visualize DNA polymorphisms between samples. These fingerprints may be used as a tool for determining the identity of specific DNA samples or to assess the relatedness between samples. Fingerprints are also used as the source for molecular markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and/or genetic loci (LEE, 1995).

a) RFLP

Restriction fragment length polymorphism (RFLP) is a DNA fingerprinting technique that is based on classical hybridisation. The technique involves the cutting of genomic DNA with restriction endonucleases followed by electrophoretic separation of the DNA fragments that are detected by Southern hybridisation with probes targeted to hypervariable regions of DNA (JEFFREYS *et al.,* 1985). This restriction cleavage allows the detection of polymorphisms in DNA fingerprints, which can result from alterations in the DNA sequence (WELSH and McCLELLAND, 1990).

The technique has widely been used for gene mapping and studying diversity in plant populations. RFLP analysis uses hybridisation technology where cloned DNA sequences are labelled using a radioactive label and used as probes to identify differences in the sizes of specific genomic DNA fragments following

digestion by a restriction endonuclease (NEWBURY and FORD-LLOYD, 1993). RFLP methodology has been shown to be costly, time consuming, difficult to establish and technically challenging, especially in species with large and complex genomes (RAGOT and HOISINGTON, 1993). As an alternative to RFLP markers which use radioactive probes, sensitive non-radioactive chemi-illuminescent detection systems can be used. The other techniques that prove to be faster and easier than RFLP are Polymerase Chain Reaction (PCR) based methods.

b) Microsatellites

This is a PCR based method that requires the use of more specific primers namely microsatellite and telomere sequences. Microsatellite repeats (also known as simple sequence repeats [SSR]) are hypervariable DNA sequences consisting of arrays of basic repeat units of two to eight base pairs (bp) probed with labelled oligonucleotides. Other people have referred to this technique as variable number of tandem repeats (VNTR) or minisatellites (NEWBURY and FORD-LLOYD, 1993).

Microsatellites bind to mono, di-, tri-, and tetra-nucleotide repeat sequences; in particular the dinucleotide repeats (AC)n, (AG)n, (AT)n have been shown to be abundant, highly polymorphic and occur randomly dispersed in the DNA in all eukaryotic genomes (JEFFREYS *et al.*, 1985). Simple sequence repeats are analysed by PCR amplification of a short genomic region containing the repeated sequences and size estimation of the repeat length by gel separation. Only a small quantity of DNA is required and agarose gels can be used for band separation but resolution of all alleles often requires the use of acrylamide gels. A minisatellite fingerprint is made by hybridisation of a labelled probe to a Southern blot. The fingerprint obtained may contain numerous reproducible polymorphisms in one lane but the amount of work involved makes detection of microsatellite containing DNA more time consuming than amplification with RAPD (randomly amplified polymorphic DNA) (RAFALSKI and TINGEY, 1993).

c) RAPD

The RAPD technique is an application of PCR and depends on the heat-stable enzyme *Taq* polymerase, which is extracted from an organism that lives in hot springs, *Thermus aquaticus*. The enzyme is thus thermostable and hence it works optimally at 72 °C (NEWBURY and FORD-LLOYD, 1993).

The method utilizes short primers of arbitrary nucleotide sequences that are annealed in the first few cycles of PCR at low stringency. This stringency of the early cycles ensures the generation of products by allowing priming with mismatches between primer and template. The subsequent PCR cycles are performed at a higher stringency after the generation of some initial PCR products that now have ends complementary to the primers (NEWTON and GRAHAM, 1994).

When the temperature is raised to 94 °C, the strands of the genomic DNA separate. Lowering the temperature to ± 45 °C (but depending on the size and sequence of primer) allows the primers to anneal to the specific regions of the template DNA. Raising the temperature to 72 °C, removes all those primers that are not tightly hybridised and so helps prevent the copying of portions of the genome that are not bound by primers. The enzyme can only start to amplify from a double stranded piece of DNA and this is provided by the primer-genomic DNA duplex. DNA sequences have polarity and thus the enzyme will only amplify the DNA in one direction (NEWBURY and FORD-LLOYD, 1993).

The results of PCR are viewed on a stained electrophoresis gel. The bands obtained in the gel serve as raw data for comparison of plant genotypes. In some cases ethidium bromide-stained agarose gels and in others, silver-stained polyacrylamide gels have been used to separate and visualise amplification products. The banding patterns are repeatable for a plant genotype using a

particular primer provided that all of the reaction characteristics are consistent (NEWBURY and FORD-LLOYD, 1993).

For any PCR reaction to work the following are required: DNA template, DNA *Taq* polymerase, dinucleotriphosphates (dNTPs), primers and buffers. The DNA template is the DNA strand to be amplified while *Taq* polymerase is the heat-stable enzyme that utilizes dNTPs to build up a complementary DNA strand. The primer is a short oligonucleotide sequence that binds to the template at specific complementary sites. This marks the start for amplification and guides the *Taq* polymerase. The PCR buffer also contains $MgCl_2$. The Mg ions form a soluble complex with the dNTPs, essential for dNTP incorporation. In addition, the Mg ions stimulate polymerase activity and increase the temperature at which correctly base-paired DNA and primer-template interactions dissociate (TAYLOR, 1998).

RAPD is a technically simpler technique compared to RFLP and microsatellites. RAPD comprises several steps including plant DNA extraction, an assessment of the DNA concentration, incubation of several components in a computerized thermal cycler and finally gel electrophoresis. RFLP on the other hand, requires more steps. These are: a) plant DNA extraction; b) restriction enzyme digestion; c) gel electrophoresis; d) blotting onto a membrane; e) preparation of a radioactively-labelled probe; f) hybridization and filter washing; and, g) autoradiography. Both the techniques require ultra-pure DNA. RAPD usually requires very small quantities of starting DNA material. Variations detected by RAPD primers are relatively higher than the variations detected by RFLP probes (NEWBURY and FORD-LLOYD, 1993).

There are a number of disadvantages of RAPD analysis. If by any chance foreign DNA contaminates the reaction mixture, for example, fungal DNA sequences, those extra DNA sequences will also be amplified. They compete for binding to the primer with the actual DNA that is meant to bind to the primer and be amplified. These contaminating sequences would then be present at a lower

concentration than the amplifiable sequences in the DNA sample and in the face of competition for primers may not amplify to sufficient levels to be visible as a band in the gel at the end of the PCR (NEWBURY and FORD-LLOYD, 1993).

In fingerprinting applications, RAPD can sometimes be inappropriate especially when the polymorphism between the genomes being studied is limited to an extremely small genomic fraction (generally less than 0.1 %). For this reason, other DNA fingerprinting techniques such as DNA amplified fingerprinting (DAF) and amplified fragment length polymorphism (AFLP) are used (VOS *et al.,* 1995). The reproducibility of RAPD analysis is one of its greatest disadvantages.

d) AFLP

Amplified Fragment Length Polymorphism (AFLP) also known as selective restriction fragment amplification (SRFA) is another favoured technique for fingerprinting genomic DNA (VOS *et al.,* 1995). AFLPs are based on the selective amplification of a subset of genomic restriction fragment using PCR. DNA is digested with restriction endonucleases and double stranded DNA adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. Thus the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR (VOS *et al.,* 1995).

AFLPs have proven to be a reliable and reproducible technique. The DNA polymorphisms identified are typically inherited in Mendelian fashion and therefore may be used for typing, identification of molecular markers and mapping of genetic loci (VOS *et al.,* 1995). AFLP and RFLP are highly sensitive and costly compared to RAPDs and furthermore are time consuming. The technique involves digestion with restriction endonucleases, ligation of adapters, amplification and finally sequencing. RAPD was chosen over the other techniques for the purpose of this study.

1.9 Aims of this study

A number of *Dierama* species: *D. nixonianum, D. luteoalbidum, D. erectum, D. pallidum* and *D. pumilum* are endemic to KwaZulu-Natal. They are vulnerable in their natural habitats due to grassland transformation by agriculture and forestry. There is a need to urgently conserve these species (SCOTT-SHAW, 1999).

The aim of this study, therefore, was to establish an *in vitro* protocol for the conservation of endangered *Dierama* species namely: *D. luteoalbidum* and *D. erectum*. RAPD analysis was used to study the genetic fidelity of the plants produced *in vitro.*

CHAPTER 2: EXPLANT SELECTION AND DECONTAMINATION

2.1 INTRODUCTION

In plant tissue culture an explant is defined as the starting material from which plantlets can be derived. This derivation goes hand in hand with a theory called totipotency that states that an individual cell has the ability to regenerate into a whole organism (ALLAN, 1991). This is a normal characteristic of all plant cells although it may differ from tissue to tissue. MURASHIGE (1976) mentioned that it is essential to choose a suitable explant carefully since factors such as size, origin and physiological condition of the explant may affect their response *in vitro*. The types of explant that are normally used include: a) leaf pieces, b) ovaries, c) anthers, d) inflorescence stems, e) bulbs, corms and rhizomes and f) seeds (GEORGE, 1993). In the micropropagation of Iridaceae species, bulbs or corms and inflorescence stems are selected because they are highly regenerative and give rise to plantlets or callus (HUSSEY, 1975). Explants are also chosen based on the aim of the experiment. For example, if the aim is to produce haploid progeny, anthers are suitable explants whereas for clonal purposes bulbs, corms and rhizomes are preferred.

Once the explant has been selected it then has to be decontaminated (if not yet free of contamination, as with shoot tips). The nature of the explant may complicate its handling and sterilization (ALLAN, 1991). Problems are encountered when sterilizing stem tissues of woody species, as they are often difficult to handle and the seed coats of some species may have to be removed (ALLAN, 1991). It is even more difficult to sterilize underground explants that contain soil borne micro-organisms (HOL and VAN DER LINDE, 1992). So far leaves are amongst the easiest explants to decontaminate (ALLAN, 1991).

Surface sterilization can be carried out with several germicide reagents that are cost effective and non toxic to both plant cells and the worker. The most

commonly used are hypochloride ions and alcohols (GEORGE, 1993). For a surface sterilant to be effective it is advisable to add a detergent that acts as a wetting agent (GEORGE, 1993). A few drops of Tween-20™ are normally used. This enables the sterilant to penetrate the tissue being decontaminated. The potential germicidal activity of hypochloride solutions is due to the oxidizing capacity of their hypochlorous acid (HOCl) and the OCl^- ion. It is the OCl^- ion that is much more active than the acid and this activity decreases with an increasing pH. For effective disinfection, hypochloride solutions should be used at pH 6-7 (GEORGE, 1993). Commercial bleaches are available and are normally used as sources of hypochloride. For example, JIK contains 3.5 % sodium hypochloride (NaOCl). Some workers have found it to be more effective than calcium hypochloride (CaOCl) (GEORGE, 1993).

Amongst the alcohols, ethanol is the most widely used although it is noted that only in few cases can ethanol be used on its own to disinfect plant tissues (GEORGE, 1993). Methanol and isopropanol are less germicidal than ethanol and it has been recorded that they increase the risk of phytotoxicity (GEORGE, 1993). Alcohols are not only germicidal but also remove surface waxes from plant tissue. A preliminary dip in 70-95 % ethanol permits plant tissues to be more effectively wetted and penetrated by the other sterilants (GEORGE, 1993).

Heavy metal ions such as mercuric chloride can also be used to disinfect plant tissues. They are highly toxic and thus should be used with care. They are environmentally unacceptable and thus their use is not recommended. They are used in disinfecting soil borne explants (UPFOLD, VAN STADEN and EDWARDS, 1992). Other oxidizing sterilants that have antimicrobial activity are hydrogen peroxide and potassium permanganate, although they are not widely used (GEORGE, 1993). Fungicides such as Benlate and Dithane can also be used to reduce fungal contamination. HOL and VAN DER LINDE (1992) reported that hot water treatment prior to disinfection with sodium hypochloride greatly reduced endogenous contamination in underground organs. Antibiotics such as streptomycin and penicillin can be used to reduce microbial infection in cultures that have already been established (GEORGE, 1993).

There are several possible sources of contamination in tissue culture: a) the explant itself, b) the growth vessels, c) the growth medium, d) the growth environment and e) the instruments used in handling tissues (ALLAN, 1991). To avoid direct contamination, laminar flow benches are used. The surfaces of the benches are wiped with absolute alcohol while the hands of the worker plus all equipments placed on the bench are sprayed with 70 % alcohol. The growth medium is autoclaved at high pressure (121 Pa) and temperature (100 °C) for 20 minutes. Filter sterilization is also used in cases where the medium contains temperature sensitive substances (e.g. vitamins). The handling instruments can be sterilized with a glass bead sterilizing unit or an alcohol flame. The necks of the culture jars and tubes can be flamed to minimize contamination.

In order to successfully establish *Dierama* species *in vitro,* it was first necessary to develop a decontamination protocol using corms and seeds.

2.2 GENERAL MATERIALS AND METHODS

a) Preparation of the growth medium

Growth was initiated using the full strength MURASHIGE and SKOOG (MS) (1962) basal medium which contains various combinations of organic and inorganic elements. In addition, myo-inositol 100 mgL^{-1} (0.01 %), sucrose 20 gL^{-1} (2 %) and Gelrite 2 gL^{-1} (0.2 %) were used. Ten ml aliquots of the medium were added to 80 x 25 mm tubes. The pH of the medium was adjusted to 5.8 prior to autoclaving at high pressure (121 Pa) and temperature (100 °C) for 20 minutes.

b) Aseptic techniques

The measuring cylinders, beakers, distilled water, handling instruments and glass Petri dishes were autoclaved together with the medium. The surface of the laminar flow bench was wiped with absolute alcohol. The culture jars or tubes and Petri dish canisters were sprayed with 70 % alcohol before transferring to the bench. Three sets of handling instruments were used and a bead sterilizing unit was used to sterilize them while working on the bench. The hands of the worker were washed thoroughly with tap water and thereafter sprayed with 70 % alcohol before working on the bench. After aseptically transferring the explants onto the medium, the tubes were sealed with parafilm.

c) General growth conditions

All cultures were grown in the light (16 hours light: 8 hours dark), unless stated otherwise, and at a temperature of ± 25°C. Fluorescent light bulbs were used in the growth chambers; providing a light intensity of 71 µmol $m^{-2} s^{-1}$.

2.3 DECONTAMINATION OF CORMS

a) Materials and Methods

Corms of an unidentified *Dierama sp.* were purchased from the Croft Wild Bulb Nursery (Eastern Cape, South Africa) in August 2002 and a few *D. luteoalbidum* corms were collected from the Underberg by Mr Rogan Roth. These were potted in 20.5 cm pots in the greenhouse. The soil mixture that was used was as follows; Umgeni river sand: Organic compost: Irish peat: Seedling bark: Super phosphate: Vermiculite (12:12:2:1:1:0.5) (v/v). Young corms measuring 3-6 cm in diameter were used for experimental purposes. Prior to tissue culture the leaves and roots were removed form the corms as well as the fibrous structures surrounding the corms. Two to three corms provided sufficient material for each decontamination treatment (10 replicates), that is, by dividing each corm into four

explants. In total, 25 corms were used for the decontamination experiment. JIK, 70 % ethanol (v/v), 1 % Dithane (w/v) and 1 % Sporekill (w/v) were prepared aseptically on the bench. Two water baths were set at 50 and 55 °C and corms placed in culture jars were immersed in the hot water at the different temperatures for 10, 20 and 30 minutes. At the end of each immersion interval the culture jars were aseptically transferred to the laminar flow bench. In the first treatment, corms without hot water treatment were soaked in JIK for 30 minutes. In the second treatment, corms without hot water treatment, were dipped in 70 % ethanol for one minute and then soaked in JIK for 30 minutes. In the third and fourth treatments, corms without hot water treatment were soaked in either 1 % Dithane or 1 % Sporekill for 30 minutes. Thereafter the corms were dipped in 70 % ethanol for one minute and then soaked in JIK for 30 minutes. Corms pre-treated with hot water were then dipped in 70 % ethanol for one minute and then soaked in JIK for 30 minutes.

Following decontamination, the corm explants were rinsed several times with sterile distilled water and the dead tissues were trimmed off. Each corm was cut in half and then into quarters so as to provide the four explants.

The data collected in the decontamination of corms was: a) the number of decontaminated corms; and, b) the number of viable corms after decontamination.

b) Results and Discussion

The results presented below (Table 4) indicate that the decontamination of corms was not very successful. The highest number of corms that were decontaminated was only 40 % and this was achieved when corms were treated with hot water at 50 °C for 20 minutes and at 55 °C for 30 minutes. In both cases only 20 % of the corms were viable after decontamination. More corms regenerated shoots following decontamination at 50 °C than at 55 °C. The application of the fungicides, Dithane and Sporekill, did not improve decontamination of the corms as only 12.5 % in the former and 6.3 % in the latter were successfully

decontaminated. Furthermore, none of the corms regenerated *in vitro*. Application of JIK on its own also produced a poor decontamination response. Only 6.3 % of the corms were decontaminated and none regenerated *in vitro*. Application of JIK together with 70 % ethanol gave a better response. Only 25 % of the corms were disinfected and 75 % of these regenerated *in vitro* (Table 4).

Table 4: Decontamination of *Dierama sp.* corms after 28 days

Treatments		Decontaminated corms (%)	Viable decontaminated corms (%)
Pre-sterilization	**Sterilization**		
-	JIK (30 min)	6.3	0
70 % Ethanol (1 min)	JIK (30 min)	25	75
Fungicides			
1 % Dithane (30 min)	JIK (30 min)	12.5	0
1 % Sporekill (30 min)	JIK (30 min)	6.3	0
Hot Water			
50 °C (10 min)	JIK (30 min)	12.5	50
50 °C (20 min)	JIK (30 min)	40	20
50 °C (30 min)	JIK (30 min)	32	0
55 °C (10 min)	JIK (30 min)	32	25
55 °C (20 min)	JIK (30 min)	12.5	0
55 °C (30 min)	JIK (30 min)	40	20

JIK on its own was not a thorough disinfectant. Dipping the corms in 70 % ethanol and then disinfecting them with JIK improved decontamination. Ethanol aided the decontamination procedure by acting as a wetting agent and ensuring that the disinfectant thoroughly penetrated the explants (GEORGE, 1993). After 21 days in culture fungi and bacteria infected the corms even after fungicides were applied prior to decontamination. The infection was found to be internal as a result of endogenous micro-organisms. Underground organs (e.g. corms) are highly saturated with these soil borne micro-organisms. This indicated that the fungicides only surface sterilized and thus did not thoroughly disinfect the corms. HOL and VAN DER LINDE (1992) reported that utilization of hot water prior to

decontamination reduced endogenous microbial infection in *Narcissus* bulbs. In *Dierama* hot water slightly improved the decontamination but affected viability of the corms. After 21 days in culture some corms regenerated while others did not. In the case where no regeneration occurred, temperature, time of immersion and/or the disinfection procedure might have killed the corms. There is no clear indication that any of the above factors directly affected the viability. Due to the limited availability of corms and high incidence of contamination, seeds were selected for further experimental work.

2.4 DECONTAMINATION OF SEEDS

a) Materials and Methods

D. luteoalbidum seeds were collected at Pevensey (2929 DC), Underberg (South Africa) in December 2001 by Mr Rogan Roth. Eight treatments were used in the decontamination of the seeds. Since the plants are threatened and only a few seeds were available for experimental purposes, each treatment consisted of only 10 seeds. Although this sample size may not be adequate for determining statistically significant differences between treatments, it did indicate the relative success of the decontamination protocols tested. Decontamination was carried out in plastic sieves that were autoclaved prior to use together with the growth medium. JIK, half strength JIK and 70 % ethanol were aseptically prepared on the bench. In the first treatment seeds were soaked in JIK for 15 minutes and in the second treatment for 20 minutes. In the third treatment seeds were soaked in half strength JIK for 15 minutes and in the fourth treatment for 20 minutes. In the fifth treatment seeds were dipped in 70 % ethanol for one minute and soaked in JIK for 15 minutes and in the sixth treatment for 20 minutes. In the seventh treatment seeds were dipped in 70 % ethanol for one minute and soaked in half strength JIK for 15 minutes and in the eighth treatment for 20 minutes. After decontamination, the seeds were washed three times with sterile distilled water to remove any excess JIK and left to germinate. Germination was recorded every week for 28 days.

In the decontamination of seeds the data collected was: a) the number of decontaminated seeds; and, b) the number of germinated seeds.

b) Results and Discussion

The decontamination of the seeds was more successful than that of the corms. There was 100 % decontamination in all the treatments except for when the seeds were disinfected with JIK for 20 minutes and with ½ strength JIK for 15 minutes. Seeds were easier explants to disinfect. However, the overall germination response was very poor. Only 60 % of the seeds germinated when dipped in 70 % ethanol and disinfected with JIK for 15 minutes (Table 5).

Table 5: Decontamination and *in vitro* germination of *D. luteoalbidum* seeds after 28 days

Treatments		Decontaminated seeds (%)	Viable decontaminated seeds (%)
Pre-sterilization	**Sterilization**		
-	JIK (15 min)	100	30
-	JIK (20 min)	90	30
-	½ strength JIK (15 min)	90	10
-	½ strength JIK (20 min)	100	10
70 % Ethanol (1 min)	JIK (15 min)	100	60
70 % Ethanol (1 min)	JIK (20 min)	100	30
70 % Ethanol (1 min)	½ strength JIK (15 min)	100	20
70 % Ethanol (1 min)	½ strength JIK (20 min)	100	30

Seeds are indeed amongst the easiest explants to decontaminate (ALLAN, 1991) although their germination plays a vital role in their success as explants *in vitro*. *Dierama* seeds are very small and do not possess a very hard seed coat (HILLIARD and BURTT, 1991). Preliminary experiments were conducted to determine the cause of the poor germination response. The viability results with 0.1 % tetrazolium indicated that the majority of the seeds were not viable (data not included). The poor response was therefore, a result of the age of the seeds. The seeds were 21 months old when placed in culture. The strength of the disinfectant did not in any way affect germination of the seeds. The strength of the growth medium may have contributed to the low germination response. Some workers achieved better germination when the seeds were germinated on a low strength MS medium (McALISTER *et al.*, 1998).

Due to the unavailability of fresh seeds, no further work was done to improve the germination of the seeds. Attention then focussed on the proliferation of the few seedlings.

CHAPTER 3: PROLIFERATION OF SHOOTS

3.1 INTRODUCTION

Once the selected explants are decontaminated and successfully established *in* vitro, the next step is to multiply the propagules (GEORGE, 1993). This is achieved by inducing the cultures to produce multiple shoots or somatic embryos. This can be achieved in many ways; inducing proliferation of axillary buds and adventitious shoots from callus or alternatively, inducing shoots directly from explants. Shoots formed are usually divided and used as explants in further sub-cultures (GEORGE, 1993).

This proliferation stage is often made possible by supplementing the growth medium with plant growth regulators. Plant growth regulators are chemicals, naturally occurring and synthetic, that modify plant growth. There are several kinds of plant growth hormones: a) auxins; b) cytokinins; c) gibberellins; d) ethylene; and, e) abscisic acid, with auxins and cytokinins being by far the most important. These growth regulators are added to the growth medium. Growth *in vitro* is normally regulated by the interaction and balance between exogenous and endogenous growth regulators (GEORGE, 1993).

Auxins are normally used in tissue culture to promote the growth of callus, cell suspensions and to regulate morphogenesis, especially when used in conjunction with cytokinins. The most commonly used auxins are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthaleneacetic acid (NAA). Of all the auxins, 2,4-D is frequently used in the initiation and maintenance of callus and cell cultures. Since cultures maintained on 2,4-D supplemented medium often become genetically modified, some workers prefer using NAA or IAA. In suspension cultures, auxins promote cell dispersion and when included at high concentrations prevent morphogenesis but induce embryogenesis. Auxins also promote the formation of roots (GEORGE, 1993).

Cytokinins are used in tissue culture to promote shoot proliferation, embryogenic callus induction and root inhibition. The most commonly used cytokinins are kinetin, zeatin, N^6-(2-isopentyl) adenine (IPA) and 6-benzyladenine (BA). Cytokinins are thought to be necessary for cell division. In their presence they promote the synthesis of proteins involved in the process of mitosis. This is how they promote adventitious shoot formation, embryogenesis and overcome apical dominance in shoot cultures. Cytokinins also have negative effects as they may decrease the degree of rooting or bulblet formation and also may decrease the survival of plantlets when transferred from *in vitro* to the outside environment (KIM and DE HERTOGH, 1997).

Over the years, liquid culture has been used in place of solid media for the rapid multiplication of plants *in vitro*. In liquid shake medium there is a constant circular movement which provides better aeration and allows better absorption of nutrients from the medium. Aeration ensures the removal of other gases that may accumulate in the growth medium and in the culture vessels (ILAN *et al.*, 1995). The use of liquid culture for scaling-up purposes can greatly reduce intensive manual handling of plant material (ZIV, 1989) and is inexpensive compared to using solid medium (PÂQUES *et al.*, 1992).

The use of liquid media has its disadvantages. Cultures initiated from this medium frequently become hyperhydric which reduces the chances of survival *in vitro* (GEORGE, 1996). This can be prevented by: a) reducing humidity in the culture vessels; b) increasing the medium strength; c) decreasing the cytokinin concentration in the medium; and, d) immersing the shoots periodically in liquid medium to reduce the length of the sub-culturing period (GEORGE, 1996). Alternatively, meristemoid clusters can be induced by using growth retardants (ZIV and HADAR, 1991).

ZIV (1989) used liquid medium supplemented with growth retardants (daminozide, ancymidol or uniconazole) to induce massive bud aggregates or protocorms when using paclobutrazol. The protocorms were transferred to a semi-solid medium for rooting and corm induction. LILIEN-KIPNIS *et al.* (1992) used liquid culture to scale up *Nerine mansellii* inflorescence-derived explants on

2,4-D and BA supplemented MS medium. This resulted in callus-like tissue which when sub-cultured to NAA, BA and paclobutrazol supplemented liquid medium formed compact but friable meristematic clusters.

In the following experiments the effects of plant growth regulators on organogenesis were investigated for different explants. The aim was to determine which explant gave the best number of shoots and thereafter establish an effective and rapid shoot multiplication step.

3.2 GENERAL MATERIALS AND METHODS

The growth medium was prepared as outlined in Section 2.2 (a), except with minor adjustments where hormones were added. The aseptic techniques and growth conditions were as outlined in Section 2.2 (b-c). The results were statistically analysed using Analysis of Variance (ANOVA) in Genstat™ 5 (release 4.1 1993) where appropriate.

3.3 EXPLANT SOURCE

a) Corms

i) Materials and Methods

Following decontamination, the corms were placed on MS medium with no hormones and were carefully monitored for a period of 28 days.

ii) Results and Discussion

Corms readily formed shoots over a period of 28 days. However, as a consequence of high contamination, the shoots could not be sub-cultured onto a new medium. Rescuing the shoots in a weak solution of JIK led to shoot death and eventually the corms no longer formed shoots. On average, each quarter of

a corm of approximately 2 cm, gave rise to a shoot that developed from the lateral nodes (buds). This was as expected since this is the meristematic region of the corm (WAGNER, 1984). A better decontamination of the corms could help yield *in vitro* shoots that could be further used in the proliferation of propagules. In *D. latifolium* where corm explants were used, optimum shoot formation occurred in MS medium either devoid of hormones or supplemented with 0.5 mgL^{-1} NAA (PAGE and VAN STADEN, 1985).

b) Seeds

i) Whole seeds

1) Materials and Methods

Three-month old *D. erectum* seeds were purchased from the Silverhill Seeds Nursery (Cape Town, South Africa). The seeds were decontaminated using 70 % ethanol for one minute and JIK for 15 minutes. The seeds were germinated on a medium either devoid of hormones or supplemented with 0.5 mgL^{-1} BA. Each treatment consisted of five explants and was replicated five times. The results recorded after 28 days were as follows: a) the number of seeds that germinated and b) the number of shoots per seedling.

2) Results and Discussion

A high germination response was observed in the medium devoid of BA but more shoots were formed in the BA supplemented medium. There was no significant difference in the production of shoots in the two types of media (Table 6).

Table 6: The effect of BA on seed germination and multiple shoot formation in *D. erectum* seeds after 28 days

Treatment	Seed germination (%)	Average number of shoots/seed
MS	100	3.4 ± 0.4 a
MS + 0.5 mgL^{-1} BA	64	4.7 ± 0.6 a

LSD (5 %) = 1.4

Treatments with different letters are significantly different at $P < 0.05$

The effect of BA on seed germination was evident and the results suggested that the presence of BA in the medium inhibited germination. GEORGE (1993) reported that multiple shoots from seeds can be induced by aseptically germinating the seeds on a high cytokinin medium. Cytokinins are known to have a profound stimulatory effect on DNA metabolism and cytokinesis (VAN STADEN and DAVEY, 1979). This is a possible reason why cytokinins stimulated multiple shoot formation during seed germination. The number of shoots produced per seed for both treatments did not differ significantly between the two types of media. It is not very clear why there was a lower germination response in the BA supplemented medium but perhaps the concentration of BA that was applied was responsible for this. Since germination is very critical when using seeds as explants it would be advisable to germinate *Dierama* seeds *in vitro* in a medium devoid of any hormones and then place them on a medium containing 0.5 mgL^{-1} BA for multiple shoot formation.

ii) Hypocotyls

1) Materials and Methods

Decontaminated seedlings 6 cm in height were cut into hypocotyl and leaf explants and trimmed to about 2 cm. The following hormone concentrations were used: a) 0.5 mgL^{-1} BA, b) 1.0 mgL^{-1} BA, c) 0.5 mgL^{-1} BA: 1.0 mgL^{-1} NAA, d) 1.0 mgL^{-1} BA: 1.0 mgL^{-1} NAA, e) 1.0 mgL^{-1} NAA and f) contained no hormones. Each treatment was replicated five times due to small amount of plant material. The

results obtained after six weeks were as follows: a) frequency of shooting and rooting; and, b) callus formation. The secondary shoots obtained were used to statistically verify the results. The hormone concentrations used were as above except that an additional concentration, 2.0 mgL^{-1} BA was included. The results obtained after six weeks were statistically analysed.

2) Results and Discussion

As there was no growth response using leaf explants, hypocotyl explants were used for further experimentation. Shoot formation occurred in all treatments although at different frequencies. The highest frequency of shooting was at 0.5 mgL^{-1} BA. Increasing the BA concentration to 1.0 mgL^{-1} and including 1.0 mgL^{-1} NAA led to callus formation. Rooting occurred best in a medium devoid of any hormones (Table 7).

Table 7: The effects of BA and NAA on multiple shoot formation from hypocotyl explants of *D. luteoalbidum* after six weeks

Treatments (mgL^{-1})		Shoots (%)	Roots (%)	Callus formation
BA	NAA			
0	0	80	100	-
0.5	0	100	60	-
1.0	0	40	50	+
0	1.0	60	33.3	+
0.5	1.0	60	0	+
1.0	1.0	20	0	+

+/- indicate production or absence of callus

The results were confirmed with the secondary shoots. A higher number of shoots (an average of 4.2) was obtained using media containing 0.5 mgL^{-1} BA. Fewer shoots were produced as the concentration of BA was increased. The other growth responses of the secondary shoots (callus and roots) were similar to those of the hypocotyls (Table 8).

Table 8: The effects of BA and NAA on multiple shoot formation from secondary shoots of *D. luteoalbidum* after six weeks

Treatments (mgL^{-1})		Average number of shoots per explant
BA	NAA	
0	0	1.7 ± 0.4 b
0.5	0	4.2 ± 0.9 a
1.0	0	3.8 ± 0.9 a
2.0	0	2.7 ± 1.0 ab
0.5	1.0	1.4 ± 0.3 b
1.0	1.0	1.6 ± 0.4 b
2.0	1.0	1.7 ± 0.5 b

LSD (5 %) = 1.3

Treatments with different letters are significantly different at $P < 0.05$

A higher number of shoots was obtained in media supplemented with 0.5 mgL^{-1} BA although there was no significant difference in the numbers of shoots produced on media containing higher levels of BA. The production of multiple shoots at this level of BA was previously reported in the literature by DE BRUYN and FERREIRA (1992) and DANTU and BHOJWANI (1995) who all worked on *Gladiolus*. In the propagation of *D. latifolium*, BA did not significantly improve shoot proliferation but at 0.5-1.0 mgL^{-1} induced multiple shoot formation (PAGE and VAN STADEN, 1985). Other workers used 1-2 mgL^{-1} BA (JÄGER *et al.*, 1998; DE BRUYN and FERREIRA, 1992) for this purpose. In *Babiana* species, 2-5 mgL^{-1} BA were used with the highest frequency of shooting at 2 mgL^{-1} BA.

Callus, which resulted from increasing the BA concentration and/or addition of NAA, reduced the number of shoots produced. This result was also reported in *Babiana* although callus formation only occurred when NAA was added (McALISTER *et al.*, 1998). The presence of NAA significantly affected the production of shoots (Table 8). Auxins are known to promote callus formation (GEORGE, 1993).

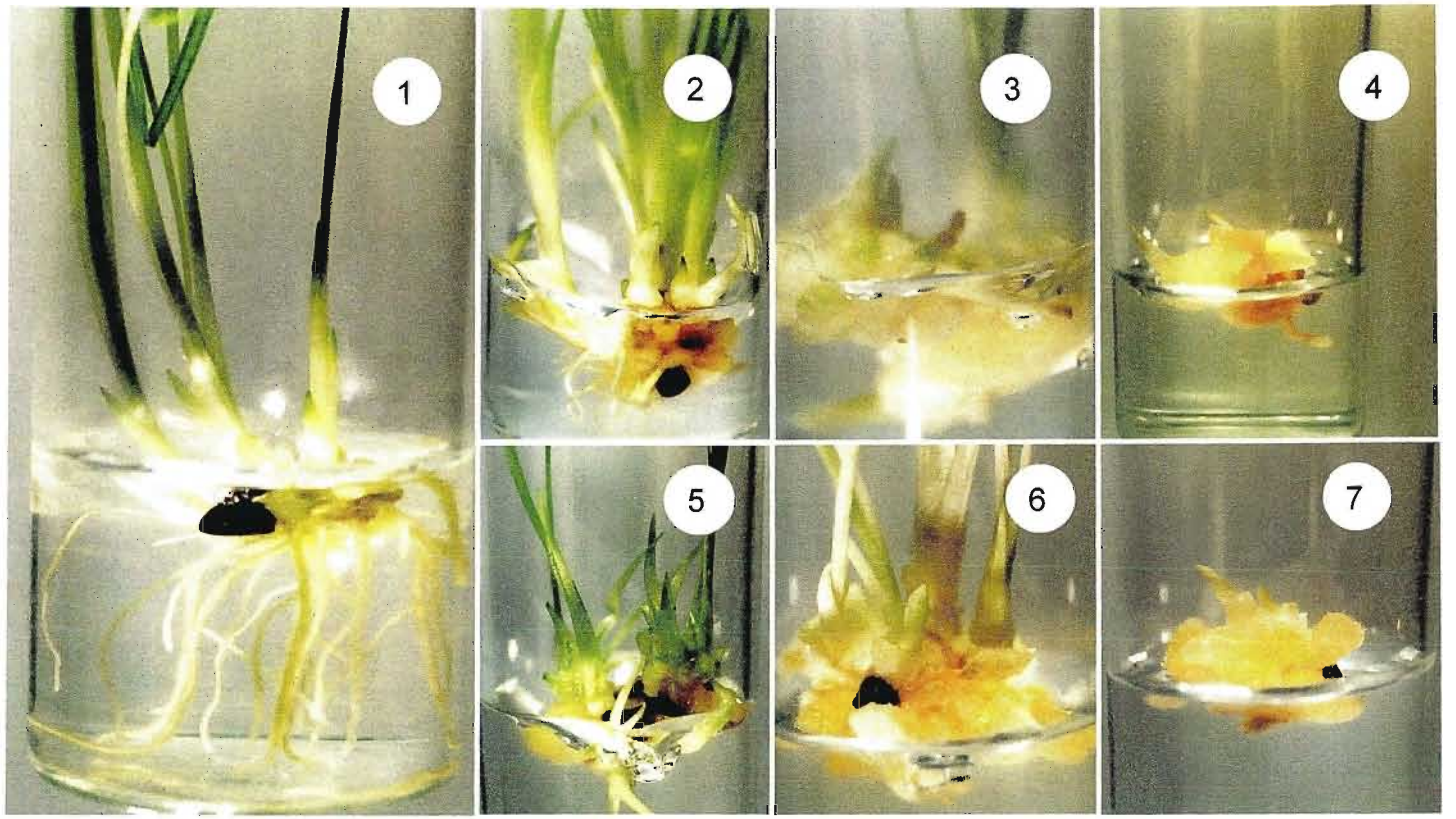

Plate 2: The effect of BA and NAA on multiple shoot formation from hypocotyls of *D. luteoalbidum* after six weeks

1) control; 2) 0.5 mgL^{-1} BA; 3) 1.0 mgL^{-1} BA; 4) 2.0 mgL^{-1} BA; 5) 0.5 mgL^{-1} BA: 1.0 mgL^{-1} NAA; 6) 1.0 mgL^{-1} BA: 1.0 mgL^{-1} NAA; and, 7) 2.0 mgL^{-1} BA: 1.0 mgL^{-1} NAA

The effect of hormones on rooting clearly indicated that rooting occurred best in a medium devoid of hormones (Plate 2). The inclusion of BA and NAA in the medium decreased the frequency of rooting (Table 7). Similar results were reported by McALISTER *et al.* (1998). Optimum rooting on a hormone-free MS medium was first demonstrated by HUSSEY (1980). Hormone-free media have been used ever since in rooting Iridaceae species (GEORGE, 1993).

Of all the explants tested, hypocotyls and seeds (germinated on BA supplemented medium), gave the highest number of shoots. Corms were difficult to disinfect and thus were not suitable to work with. Seeds on the other hand were easier to disinfect and handle while the *in vitro* grown hypocotyls were already free of contaminants. The only disadvantage of germinating seeds on BA supplemented medium was the lower germination response compared to germination on hormone-free MS medium.

After the production of multiple shoots, liquid culture was used in the multiplication of the propagules.

3.3 MASS PROPAGATION IN LIQUID MEDIUM

a) Liquid medium

i) Materials and Methods

Shoots were trimmed to about 2 cm and were aseptically transferred to 100 ml Erlenmeyer flasks or to 200 ml culture jars containing 40 ml of the liquid or solid medium respectively. The MS medium was supplemented with 0.5 mgL^{-1} BA. The flasks were sealed with cotton wool and agitated at 120 rpm in the growth room. The medium was changed every six to eight weeks and the fresh weight (FW) recorded at each sub-culture. The Growth Index (GI) was calculated at the end of each sub-culture as follows: $(FW_2-FW_1)/FW_1$.

ii) Results and Discussion

After six to eight weeks in liquid medium, the shoots gave rise to (meristemoids), semi-organized callus-like structures (Plate 3). The average fresh weight of meristemoids in liquid medium was greater than the shoot clusters in solid medium. As a result their growth index in liquid medium was greater than on solid medium.

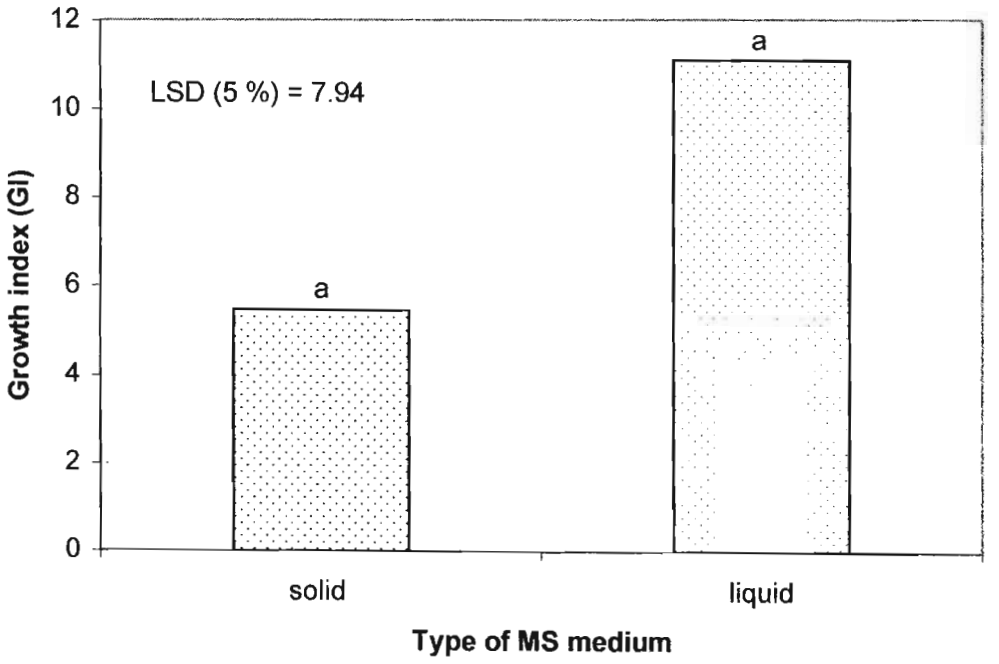

Figure 1: The growth index of *D. luteoalbidum* shoots in solid and liquid medium after six to eight weeks

Treatments with the same letters are not significantly different from each other at $P < 0.05$

The difference in the GI of the two types of media suggested that the liquid medium resulted in a better proliferation of the shoots than the solid medium. This difference was however not significant at the 95 % confidence interval. Furthermore, meristemoid clusters formed in liquid medium as opposed to shoot clusters in solid medium

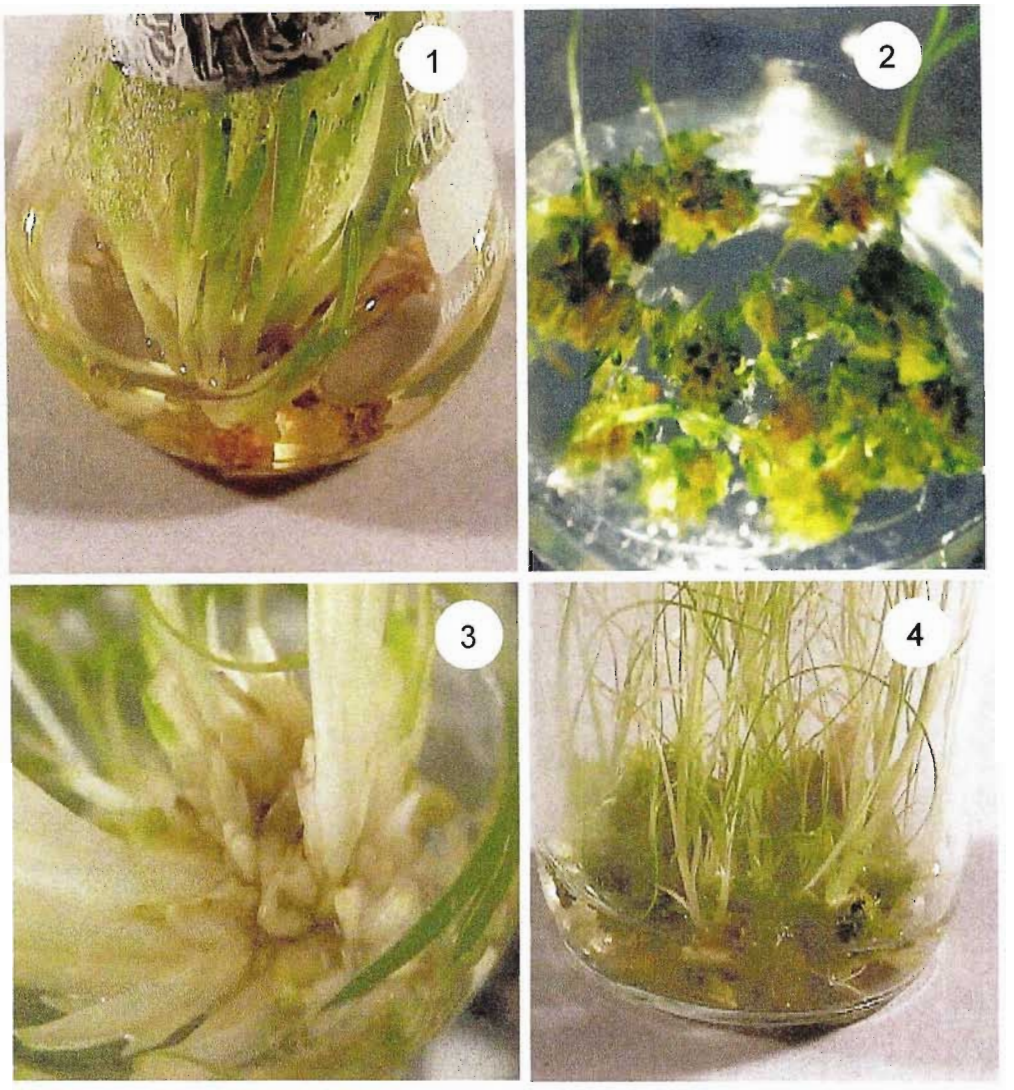

Plate 3: The effect of liquid medium supplemented with 0.5 mgL^{-1} BA on multiple shoot formation in *D. luteoalbidum* after six to eight weeks

1) multiple shoot formation from hypocotyls; 2) meristemoid induction after 8 weeks, 3) shoot induction from meristemoids; and, 4) shoot elongation from meristemoids

Liquid media is used effectively in many plants for large-scale propagation because it is inexpensive (PÂQUES *et al.,* 1992) and does not require intensive labour (GEORGE, 1996). The most common problem with liquid culture is the occurrence of hyperhydric shoots (ILAN *et al.,* 1995). Inducing meristemoid formation normally solves this problem. Most workers induced meristemoids by using growth retardants that inhibit leaf formation and promote bud aggregates (ZIV, 1989; LILIEN-KIPNIS *et al.,* 1992). In this study, the meristemoids were formed without the inclusion of growth retardants. There is a possibility that the meristemoids were induced as a result of the cytokinin in the medium and the continuous agitation. LILIEN-KIPNIS *et al.* (1992) included BA in the meristemoid-producing medium, which might indicate that cytokinins play a role in meristemoid formation. More importantly, it was the effect of growth retardants that prevented leaf development and enhanced the formation of meristemoids (LILIEN-KIPNIS *et al.,* 1992; ZIV, 1990).

Liquid medium proved to be better than solid medium for rapid multiplication. The possible reason is that liquid-shake cultures provide better aeration than stationary solid cultures. The constant circular movement provides thorough gaseous exchange for proper growth of the plant tissues (ILAN *et al.,* 1995). Furthermore, this allows for a better uptake of nutrients from the medium (ILAN *et al.,* 1995). As a result of a very low frequency of hyperhydric shoots, the medium was changed every six to eight weeks. Furthermore, transferring the meristemoids to solid medium for shoot elongation eliminated the problem of hyperhydricity.

b) Shoot elongation

i) Materials and Methods

Following sub-culture in liquid media, the meristemoid clusters were cut into manageable sizes, weighed and aseptically transferred to a shoot elongation medium. About 0.5 to 1.0 grams of meristemoid material was placed onto solid MS medium supplemented with 0.5 mgL^{-1} BA and the results were recorded after

six weeks. The number of buds was counted prior to inoculation and later the number and length of shoots produced were recorded.

ii) Results and Discussion

On average, 0.5 to 1.0 grams of the meristemoid material gave between 8 to 10 shoots and the length of the shoots varied from 50 to 65 mm after six weeks.

Table 9: The average number and length of *D. luteoalbidum* shoots (derived from the meristemoid clusters) in solid MS medium after six weeks

FW (g)	Number of initial buds	Number of elongated shoots	Frequency of shoot initiation (%)	Length of shoots (mm)
0.5	21	8.0 ± 0.9	38.1	49.8 ± 1.7
1.0	21	9.5 ± 0.9	45.2	64.5 ± 3.4

The frequency of shoot initiation was quite low. This implies that not all the buds gave rise to shoots. It appears that inoculum size had little effect on shoot production and elongation. Presumably a better shoot elongation medium could help yield more shoots per unit gram of meristemoid tissue. In *Gladiolus* cv. 'Eurovision' shoots were elongated in 30 ml liquid MS medium supplemented with 0.5 mgL^{-1} BA. The propagules were grown in 250 ml Erlenmeyer flasks in continuous light and were stationary for four weeks. From 10 buds at least six shoots, at an average length of 10.5 cm, were produced (DANTU and BHOJWANI, 1995).

In conclusion, for mass proliferation of *Dierama* shoots, hypocotyl explants can be subjected to liquid shake culture using a MS medium supplemented with 0.5 mgL^{-1} BA. Thereafter the meristemoid clusters can be transferred to solid medium supplemented with 0.5 mgL^{-1} BA for shoot elongation. The shoots can then be rooted or be used as explants for further propagation of this species. This emphasizes the fact that *in vitro* techniques can provide a reserve of propagules at a much faster rate than conventional propagation techniques.

CHAPTER 4: ROOTING, CORM INDUCTION AND ACCLIMATIZATION

4.1 INTRODUCTION

The rooting stage is necessary prior to transferring plants to the external environment. Therefore, the elongated shoots derived from the meristemoids had to be aseptically transferred to a different medium for root formation and subsequently storage organ formation prior to transferring them to the external environment (GEORGE, 1993).

Rooting in the Iridaceae occurs readily. In *Dierama latifolium*, single shoots were rooted on a hormone-free MS medium or supplemented with 0.5 mgL^{-1} NAA (PAGE and VAN STADEN, 1985). *Gladiolus* cv. 'Friendship' shoots were also rooted on hormone-free and auxin-supplemented MS media (DANTU and BHOJWANI, 1995). *Gladiolus carneus* shoots were rooted on only hormone-free MS medium (JÄGER *et al.*, 1998). Similar results were achieved for the micropropagation of *Babiana* species (McALISTER *et al.*, 1998). It was reported that some other *Gladiolus* species formed roots on ½ strength MS medium supplemented with 0.2-0.5 mgL^{-1} BA and others on a medium containing auxin and activated charcoal (GEORGE, 1996).

Activated charcoal is used in tissue culture to either inhibit or promote growth. It has a high adsorptive capacity that adsorbs inhibitory substances, regulatory hormones and other organic compounds (PAN and VAN STADEN, 1998). Its functions *in vitro* include: promotion of rooting, shooting, callus formation and growth of somatic embryos (PAN and VAN STADEN, 1998).

Activated charcoal provides an acidic environment necessary for the complete hydrolysis of sucrose into glucose and fructose (PAN and VAN STADEN, 1998). Sugar is an important constituent of the nutrient medium; it serves as an energy source for the growing tissue *in vitro* (VAN AARTRIJK and BLOM-

BARNHOORN, 1980). Sucrose is stored in the form of starch and is found in abundance in corms and bulbs (VISHNEVETSKY *et al.*, 2000). Sucrose added to a growth medium promotes organogenesis and only at extremely elevated levels can it inhibit growth. In *Lilium speciosum* 'Rubrum' sucrose concentrations from 3-5 % led to an increased number of plantlets per explant and bulblet weight of those plantlets. Shoot formation took place also on a medium devoid of sucrose due to the endogenous sucrose in the explant tissue (VAN AARTRIJK and BLOM-BARNHOORN, 1980).

Sucrose is not only used for organogenesis in tissue culture but it is also used for controlling the water potential of the growth medium (BROWN *et al.*, 1979). Mild osmotic stress in plant tissues affects cellular morphology. Sucrose crosses the cell membrane and penetrates the tissues creating a critical turgor pressure needed before cell growth (expansion) can occur (BROWN *et al.*, 1979).

There are advantages to producing storage organs *in vitro* in ornamental plants that normally produce bulbs and corms *ex vitro*. The bulblets or cormlets produced *in vitro* can be subdivided to provide secondary explants for further propagation and are beneficial for planting *ex vitro* as they do not require special acclimatization routines (GEORGE, 1996). Acclimatization is an important stage in tissue culture as a loss of plant material at this stage has serious financial implications (LINDSEY *et al.*, 1998).

In vitro corm formation has been achieved for many *Gladiolus* species. The most frequently used supplements to the growth medium were sucrose, activated charcoal, auxins and growth retardants. *Gladiolus* shoots left undisturbed in ½ (LINSMAIER and SKOOG, 1965) LS medium containing 20 gL^{-1} sucrose and 0.12-2.0 mgL^{-1} BA produced small corms (HUSSEY, 1977b; GEORGE 1996). Corm induction was enhanced by adding 60-90 gL^{-1} sucrose to MS medium (DE BRUYN and FERREIRA, 1992). *Gladiolus* was rooted on a medium containing 60 gL^{-1} sucrose, 0.02-1.9 mgL^{-1} NAA and 0.5-5 gL^{-1} activated charcoal and later formed corms (LILIEN-KIPNIS *et al.*, 1992a). Corm induction was successfully achieved in liquid medium supplemented with high levels of sucrose (DANTU and BHOJWANI, 1995; ZHOU *et al.*, 1999). At least up to 10 % sucrose was

used in taro (ZHOU *et al.*, 1999) and optimally at 6 % in *Gladiolus* (DANTU and BHOJWANI, 1995).

There are several reports on the usage of growth retardants in conjunction with liquid medium for corm formation. *Gladiolus* corm formation was promoted by treating bud and shoot explants with paclobutrazol (ZIV, 1989; STEINITZ *et al.*, 1991). Paclobutrazol was widely used in other species; *e.g.* in taro (ZHOU *et al.*, 1999) for corm induction and in potato (ŠIMKO, 1993) for tuberization. The growth retardants interfere with gibberellin biosynthesis and hence are often referred to as anti-gibberellins (KOEN, 2001).

In the following experiments the effects of sucrose, activated charcoal and paclobutrazol on rooting and corm formation were investigated. The rooted shoots were then transferred to the greenhouse for acclimatization.

4.2 GENERAL MATERIALS AND METHODS

The growth medium was prepared as outlined in Section 2.2 (a), except for minor adjustments to levels of sucrose, activated charcoal and paclobutrazol. The aseptic techniques and growth conditions were as outlined in Section 2.2 (b-c). The results were statistically analysed with Analysis of Variance (ANOVA) in Genstat™ 5 release 4.1 (1993) where appropriate.

4.3 EFFECT OF SUCROSE AND ACTIVATED CHARCOAL ON ROOTING AND CORM INDUCTION

a) Materials and Methods

Once the shoots from the elongation medium had reached a height of 6 cm they were trimmed to about 2 cm and transferred to the growth medium. The medium was supplemented with different concentrations of sucrose (2-8 %) with or without 5 gL^{-1} activated charcoal. Each treatment consisted of five explants and

was replicated five times. The number of roots and corms was recorded after 12 weeks and six months, respectively.

b) Results and Discussion

Rooting occurred in all the treatments after three months although at different frequencies. As the concentration of sucrose increased so did the number of roots produced per explant (Figure 2). More roots were produced in charcoal-supplemented medium, although this was not significantly different from roots produced in charcoal-free medium. The addition of activated charcoal only had a significant impact on root length at the 6 % sucrose level (Figure 3).

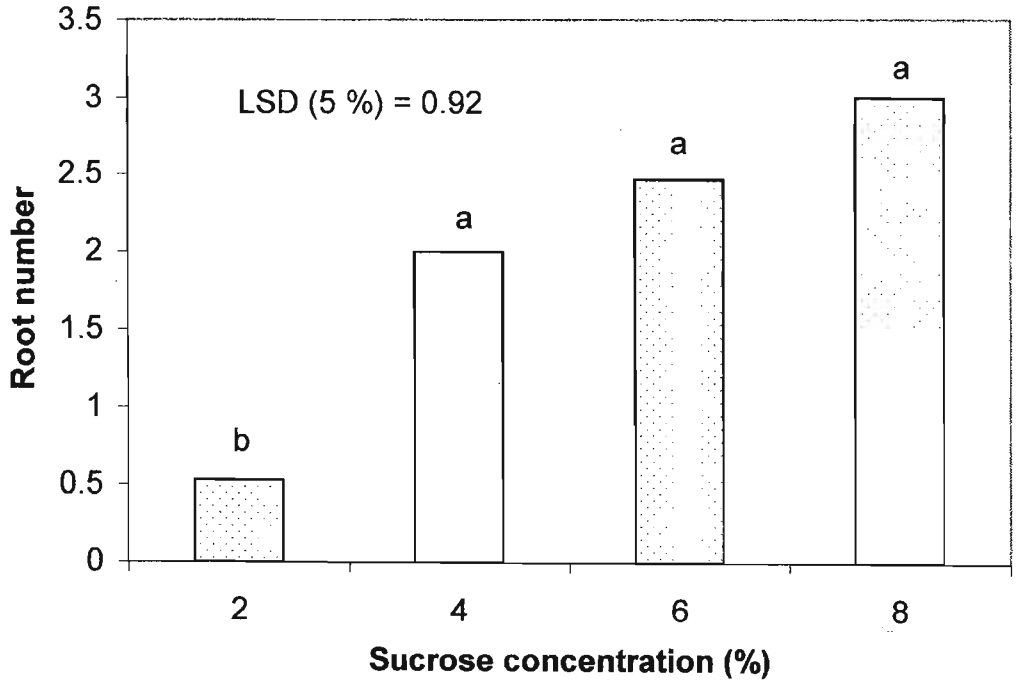

Figure 2: The effect of sucrose on the production of *D. luteoalbidum* roots after three months

Treatments with different letters are significantly different at $P < 0.05$

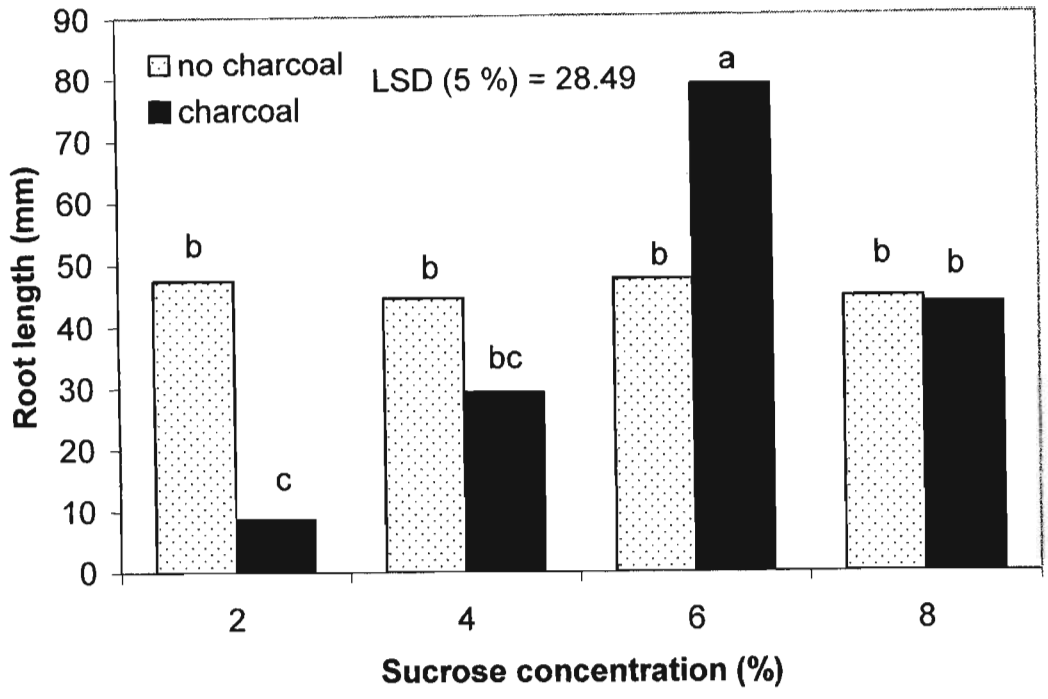

Figure 3: The effect sucrose and activated charcoal on the length of *D. luteoalbidum* roots after 3 months

Treatments with different letters are significantly different at $P < 0.05$

The results above agree with previous reports in the literature. While other *Gladiolus* species required an auxin supplement for rooting (DANTU and BHOJWANI, 1995), *D. luteoalbidum* shoots were rooted on hormone-free MS medium supplemented with 2-8 % sucrose and/or 5 gL^{-1} activated charcoal. Hormone-free MS medium was also used in the rooting of *D. latifolium* shoots although charcoal was omitted and 3 % sucrose was used. Sucrose is an important constituent of the growth medium which acts as an energy source to promote organogenesis (VAN AARTRIJK and BLOM-BARNHOORN, 1980). The higher the sucrose concentration, the higher the number of roots formed. The interaction between sucrose and charcoal produced longer roots at the 6 % sucrose level. This is due to the hydrolytic property of activated charcoal. It provides the correct pH necessary for complete hydrolysis of sucrose to more soluble forms of glucose and fructose (PAGE and VAN STADEN, 1998). These are then easily absorbed from the medium and transported to the growing tissues (BROWN *et al.*, 1979). The sucrose in the growth medium was used in the formation of roots.

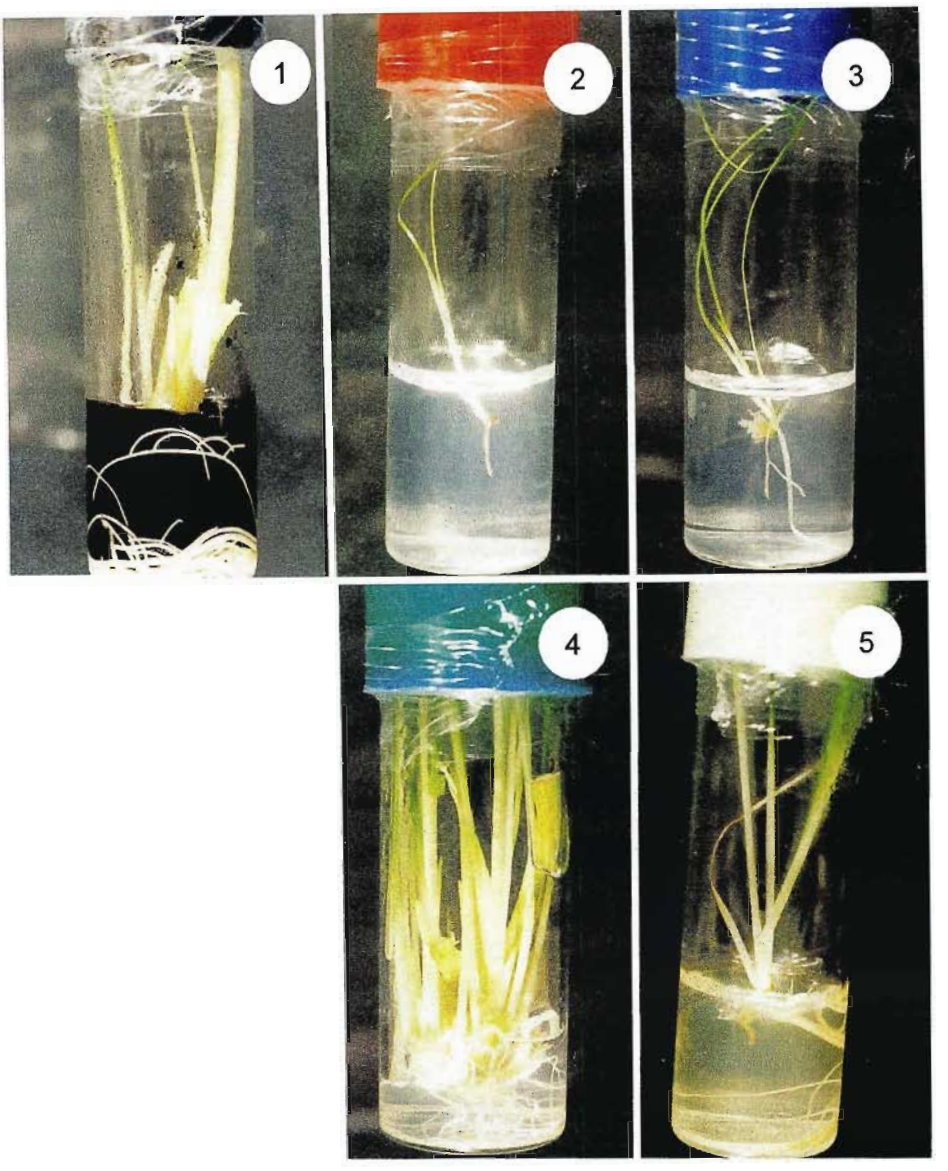

Plate 4: The effect of sucrose and activated charcoal on the rooting of *D. luteoalbidum* after three months

1) increased root length on media containing 5 gL^{-1} activated charcoal; 2) rooting on 2 % sucrose; 3) rooting on 4 % sucrose; 4) rooting and multiple shoot formation on 6 % sucrose; and, 5) rooting on 8 % sucrose

The rooted shoots left undisturbed for six months in the same medium formed small cormlets at the base of the shoots. The formation of corms only occurred at high levels of sucrose, mostly at 8 % sucrose (Figure 4). There was a significant difference in corm production between sucrose and activated charcoal at the 8 % sucrose level.

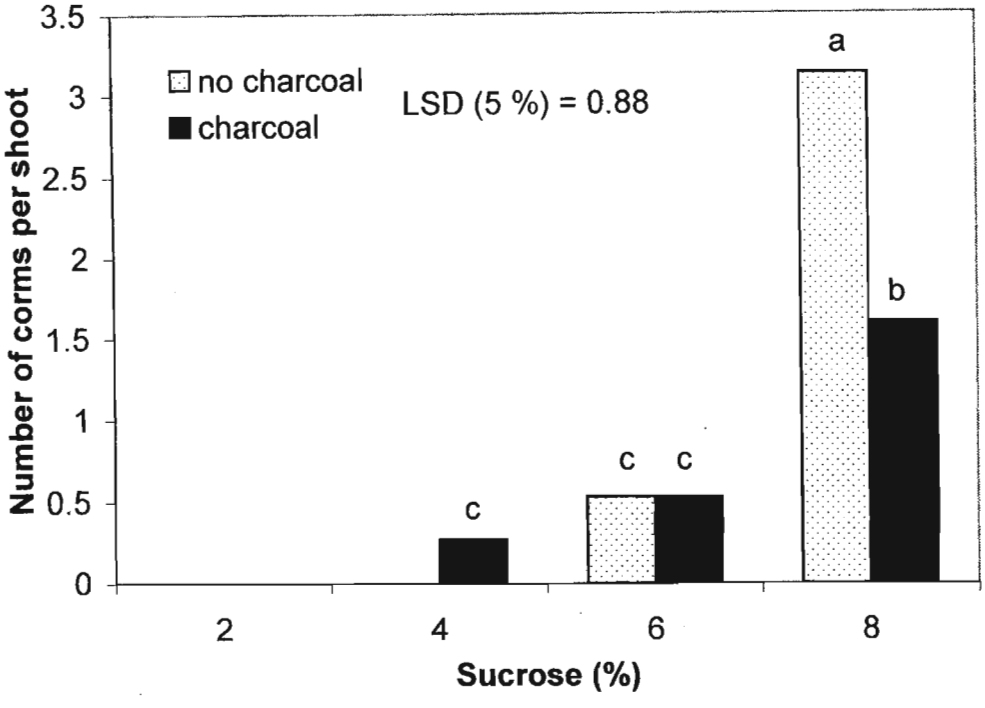

Figure 4: The effect of sucrose and activated charcoal on *in vitro* corm formation of *D. luteoalbidum* after six months

Treatments with different letters are significantly different at $P < 0.05$

Plants are unique in that they switch towards storage organ formation as a protective mechanism that sustains life even when unfavourable conditions arise. Starch, which is one form of storing carbohydrates, is found in abundance in these organs (VISHNEVETSKY *et al.*, 2000). Plants use starch as an energy source for vegetative propagation (*i.e.* annual corm formation).

Multiple shoots formed alongside the corms. This may have been due to the fact that sucrose is found in abundance in the growing regions of the tissues and is used to promote shoot formation (VAN AARTRIJK and BLOM-BARNHOORN, 1980). Higher sucrose levels promote corm formation in many species. Multiple shoot formation in *Gladiolus* was enhanced by adding 6-9 % sucrose to the

shoot induction medium. The same medium was optimal for corm production (DE BRUYN and FERREIRA, 1992). Optimal corm production on liquid MS medium containing 6 % sucrose was reported in *Gladiolus* (DANTU and BHOJWANI, 1995) in taro using 8-10 % sucrose (ZHOU *et al.*, 1999) and in potato for tuberization using 8 % sucrose (ŠIMKO, 1993).

Activated charcoal clearly did not have a beneficial effect on the production of corms in this study. The inclusion of activated charcoal reduced both corm formation and growth by at least 25 % in *Gladiolus* (DANTU and BHOJWANI, 1995). It is thus not very useful to add activated charcoal to the rooting and corm induction media. It could be interesting to see what effect the *in vitro* formed corms would have on the survival of *Dierama* plantlets *ex vitro.*

4.4 EFFECT OF PACLOBUTRAZOL ON ROOTING AND CORM INDUCTION

a) Materials and Methods

Shoots from the elongation medium were isolated, trimmed to about 2 cm and thereafter transferred to the growth medium. The medium was supplemented with 6 % sucrose and 1 to 10 mgL^{-1} paclobutrazol. Each treatment consisted of five explants and was replicated five times. The corm induction results were obtained after three months.

b) Results and Discussion

Corm formation occurred after three months in the paclobutrazol treatment. More corms were produced at 5 and 10 mgL^{-1} paclobutrazol. There was no significant difference in the production of corms at these levels. Short and thick roots were obtained only at 1.0 mgL^{-1} paclobutrazol. There were very few or no roots formed as the concentration of paclobutrazol was increased (Plate 5).

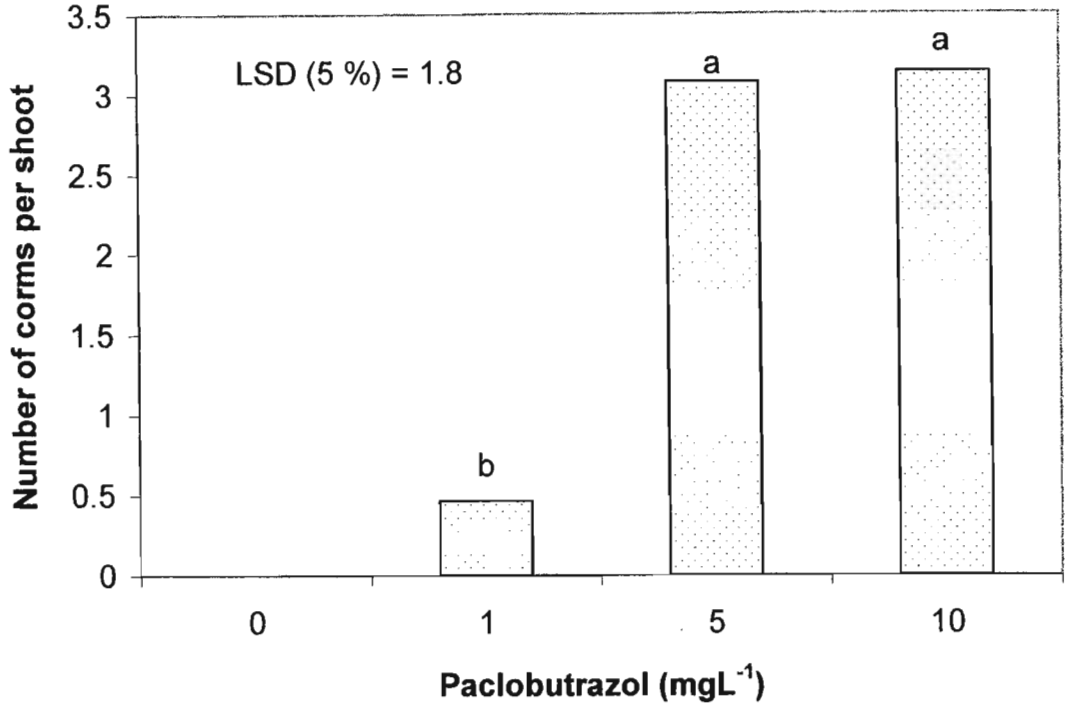

Figure 5: The effect of paclobutrazol on *in vitro* corm formation of *D. luteoalbidum* after three months

Treatments with different letters are significantly different at $P < 0.05$

The beneficial use of paclobutrazol in this instance was the shortening of the corm development period from six to three months. The number of corms produced per shoot in the paclobutrazol treatment was almost the same in the sucrose treatment. The production of cormels at the base of the shoots was a consequence of the growth retardant, paclobutrazol. Growth retardants like paclobutrazol inhibited leaf growth and led to protocorm aggregate formation in *Gladiolus* (ZIV, 1989). In *Dierama* however, leaf growth was not inhibited although root development was stunted. This was not previously reported in the literature. It is well documented that the corms of *Gladiolus* and many other corm-producing plants go through a dormant stage where the leaves completely die back. *Dierama* corms never go through that stage thus the leaves never completely die back (HILLIARD and BURTT, 1991). This is due to the fact that *Dierama* is absent from winter-rainfall habitats.

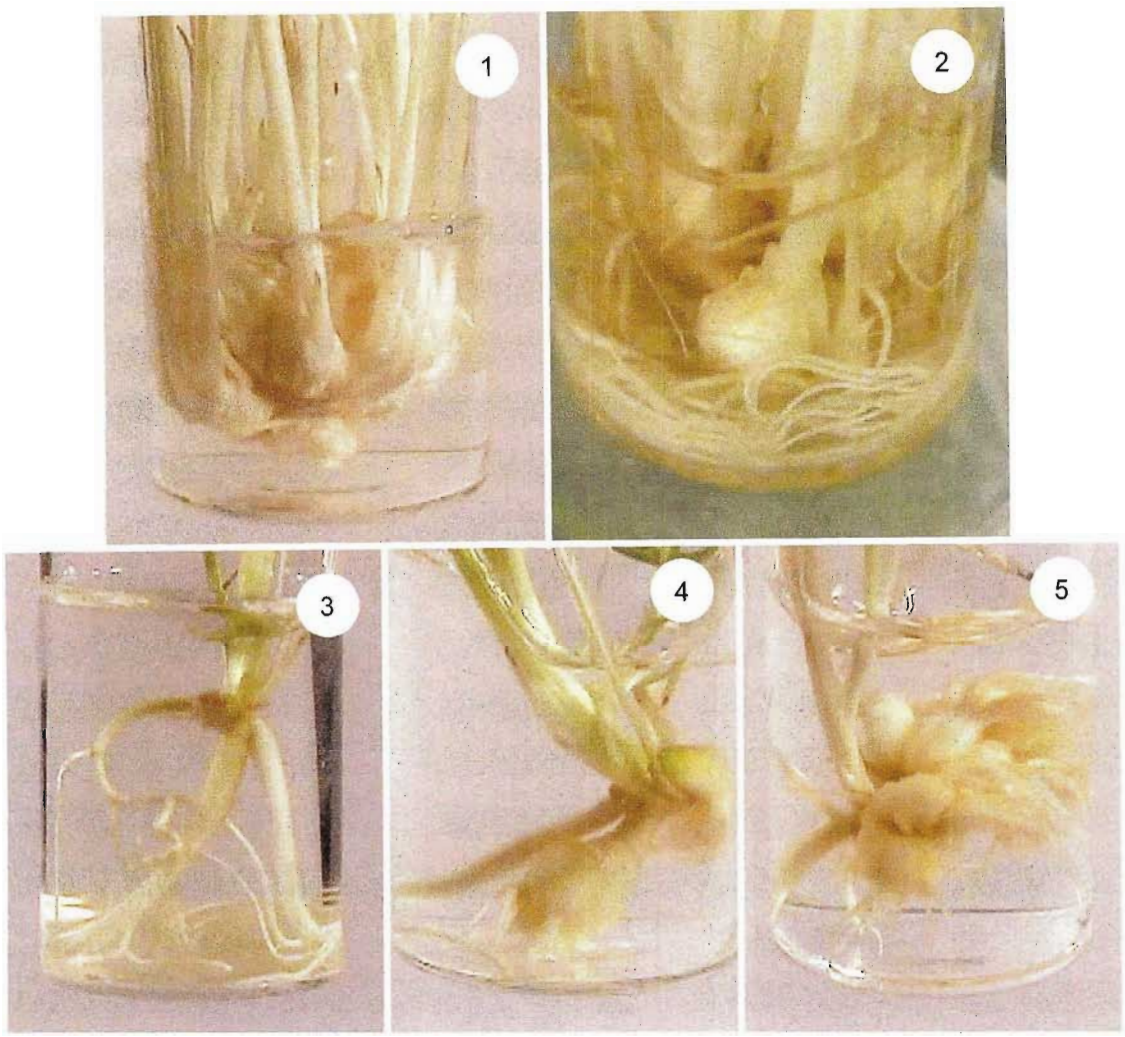

Plate 5: The effect of sucrose and paclobutrazol on *D. luteoalbidum* corm induction after six and three months respectively

1) multiple shoots with corms developing after six months; 2) corm development on 8 % sucrose after six months; 3) thick roots on 1 mgL^{-1} paclobutrazol after three months; 4) corm induction on 5 mgL^{-1} paclobutrazol after three months; and, 5) corm clusters on 10 mgL^{-1} paclobutrazol after three months

The only advantage of using paclobutrazol instead of sucrose for corm induction was for shortening the corm-development period. The production of multiple shoots which then formed roots and corms in the sucrose treatment offers an advantage over the paclobutrazol treatment. However, the formation of corm clusters in the paclobutrazol treatment could be an advantage. These corms could be used as explants for further multiplication of this species. The only disadvantage of the paclobutrazol treatment was the poor development of roots. It could have been interesting to see how plantlets from the paclobutrazol treatment would respond in the hardening process.

4.5 HARDENING-OFF

a) Materials and Methods

The shoots that had successfully formed roots were transferred to the *ex vitro* environment. Two types of soil mixtures were used: a) soil: sand: vermiculite (1:1:1) (v/v); and, b) peat: fine bark: organic compost (1:1:1) (v/v). The soil was autoclaved at 121 Pa for 20 minutes just prior to use. The plantlets were first dipped in a 0.15 % (w/v) solution of Benlate before planting out. The plantlets were kept in the mist house for three to four weeks. After this period they were transferred to the greenhouse where they were watered daily for the first week and every second day from the second week. A 1 % (v/v) solution of Kelpak was applied as a soil drench to half of the plants while the rest were watered with only tap water. After six months the plantlets were harvested and the size, fresh weight and number of the *ex vitro* formed corms were determined.

b) Results and Discussion

A total of 80 % of the plants survived in the soil: sand: vermiculite potting mixture and 100 % in the peat: compost: bark mixture after four weeks in the mist house. Three weeks after transferring the plantlets to the greenhouse only 60 % survived in the soil: sand: vermiculite potting mixture and 70 % in the peat: compost: bark mixture. The peat: compost: bark soil mixture retained more

water than the soil: sand: vermiculite. This is a possible explanation for the higher survival rate in this soil mixture. Low humidity may also be responsible for the loss of plant material in the greenhouse. Autoclaving the soil, dipping the plantlets in Benlate plus thoroughly washing-off Gelrite eliminated any possible contaminants in the roots.

On average, one corm formed in each plantlet in both the Kelpak and water treatment. The average fresh weight of the corms in the Kelpak treatment was 0.23 g and 0.18 g in the water treatment. The average diameter of the corms was 6.0 mm in both the Kelpak and water treatments (Table 10).

Table 10: The effect of Kelpak on the size, fresh weight and number of *D. luteoalbidum* corms produced *ex vitro* after six months

Treatments	Number of corms	FW (g)	Diameter (mm)
Kelpak	1.08 ± 0.08	0.23 ± 0.14	6.08 ± 0.03
Water	1.09 ± 0.09	0.18 ± 0.08	5.86 ± 0.03

The application of 1 % Kelpak did not significantly improve the production of corms *ex vitro*. Kelpak is a seaweed concentrate isolated from *Ecklonia maxima* (LINDSEY *et al.,* 1998). It contains macro- and micro-nutrients, essential vitamins and amino acids and plant regulatory hormones (KOEN, 2001). It thus has an effect on growth of plant tissues. Most workers use it to increase root growth (CROUCH and VAN STADEN, 1991; LINDSEY *et al.,* 1998). Growth in Kelpak-treated plants is regulated by the auxin: cytokinin ratio present in the solution.

In this study, 1 % Kelpak was applied to the rooted shoots for corm induction. Kelpak at concentrations between 1 and 100 % was used to successfully induce rooting in cuttings (KOEN, 2001). Applications of between 50 and 75 % Kelpak yielded the highest number of roots.

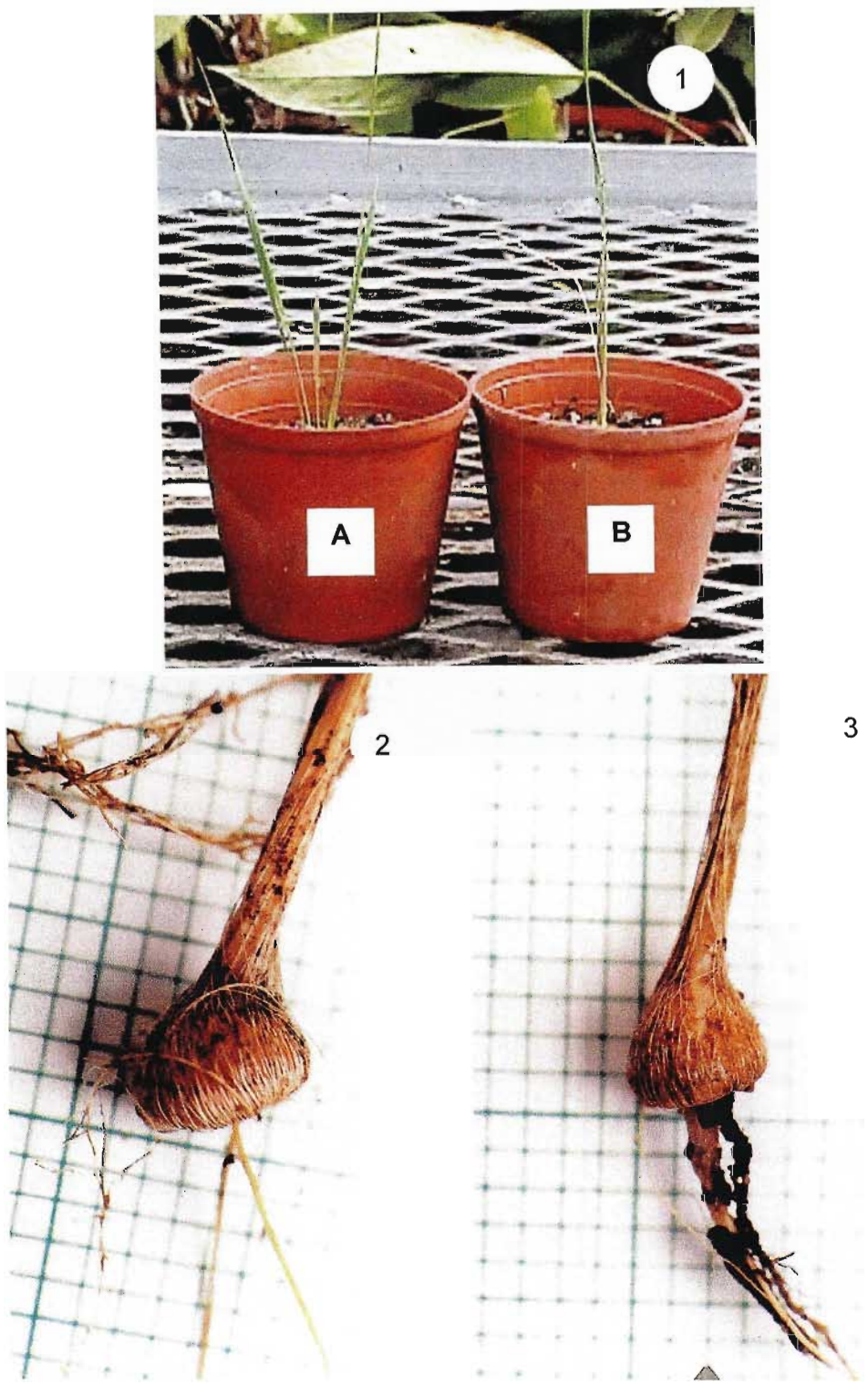

Plate 6: The effect of 1 % Kelpak on the formation of *D. luteoalbidum* corms *ex vitro* after six months

A) peat:compost:bark (1:1:1) (v/v) and **B)** soil:sand:vermiculite (1:1:1) (v/v)

1) *D. luteoalbidum* plantlets grown in the greenhouse after six months;

2) corms from Kelpak treatment; and, 3) corms from water treatment

Much lower concentrations, between 0.5 and 1.0 %, were applied by LINDSEY *et al.* (1998) and this significantly increased the growth of the shoots. In this study, the low concentration did not have any significant impact on the growth of the corms. It is possible that increasing the concentration of Kelpak might favour better production of corms. Although the effect of size of the corm on the survival of the plantlets was not investigated, it can be predicted that larger corms would survive better than smaller corms.

In conclusion, *in vitro* rooting of *Dierama* shoots occurred best on a high (8 %) sucrose MS medium after three months. Shoots left undisturbed for a further three-month period formed corms. The application of paclobutrazol yielded thick and stunted roots; and shortened the corm induction period to only three months. The plantlets in the greenhouse had a better survival on the peat: compost: bark potting mixture. The application of 1 % Kelpak did not significantly improve the acclimatization of the plantlets but yielded bigger corms.

CHAPTER 5: CLONAL FIDELITY OF THE PROPAGULES

5.1 INTRODUCTION

One of the most crucial concerns in *in vitro* propagation is to retain genetic stability in the stored germplasm (RANI *et al.,* 1995). When using tissue culture as a conservation tool, it is necessary to assess the level of genetic variation or stability in the micropropagated plants (MUNTHALI *et al.,* 1996). Tissue culture-induced variation is defined as the variation that arises *de novo* during the period of dedifferentiated cell proliferation that takes place between the culture of an explant and regeneration of plantlets (MUNTHALI *et al.,* 1996). There are two routes in the regeneration of plantlets *in vitro*; direct and indirect organogenesis (GEORGE, 1993). Direct organogenesis involves the formation of adventitious shoots or somatic embryos from differentiated cells directly on the explant (GEORGE, 1993). Indirect organogenesis involves the formation of adventitious shoots or somatic embryos from undifferentiated cells of callus (GEORGE, 1993). Callus is a tumour-like mass of cells that are unspecialised and unorganised that form in culture as a result of wounding plant tissues (DONNELLY and VIDAVER, 1988).

The actively dividing cells in callus often become genetically modified due to mutations to their DNA and changes in their ploidy level (GEORGE, 1993). Therefore, plantlets derived from callus are more likely to be genetically different from the mother plant than plantlets derived from other tissues (GEORGE, 1993). The genetic variation is genotype and explant dependent since callus derived from highly dividing 'meristematic' tissues (meristems) yield lower variation in regenerated plants than callus from other plant tissues (GEORGE, 1993).

The variability in micropropagated plants brought about by tissue culture can be detected phenotypically in the field. This process, however, takes a long time as extensive observations will only be made at a specific time or once the plant has

reached maturity (RANI *et al.,* 1995). Karyotypic analysis of metaphase chromosomes can be used to determine rearrangements and/or numerical variation in chromosomes. This process has many limitations especially in plants that have small chromosomes or too many due to polyploidy (RANI *et al.,* 1995). Isoenzyme electrophoresis can also be used (RANI *et al.*, 1995) since proteins are the direct products of individual genes (NEWBURY and FORD-LLOYD, 1993). This technique however has a limited number of informative markers and is prone to environmental and developmental variation (RANI *et al.,* 1995).

DNA based markers allow for the direct comparison of the genetic material of different plants without any environmental influence on gene expression (NEWBURY and FORD-LLOYD, 1993). The first DNA-based technique widely used for gene mapping and studying diversity in plants is RFLP (NEWBURY and FORD-LLOYD, 1993). This technique is based on restriction endonuclease digestion of genomic DNA followed by hybridization with several radioactively labelled probes that reveal polymorphisms at the DNA level (RANI *et al.,* 1995). Detection of RFLPs by Southern blot hybridization is labour-intensive (WILLIAMS *et al.,* 1990), costly and the use of radioisotopes is the major disadvantage of this technique (RANI *et al.,* 1995).

The other polymorphism assays available are based on PCR and require target DNA sequence information for the design of amplification primers (WILLIAMS *et al.,* 1990). RAPD developed by WILLIAMS *et al.* (1990) and WELSH and McCLELAND (1990), utilizes short oligonucleotide primers of arbitrary nucleotide sequence (WILLIAMS *et al.*, 1990). These primers are chosen at random without regard to the sequence of the genome to be fingerprinted (WELSH and McCLELAND, 1990). The polymorphisms detected, function as genetic markers that can be used in the construction of genetic maps (WILLIAMS *et al.,* 1990). The advantages of this technique are its speed, technical simplicity and frequency of polymorphism identification (NEWBURY and FORD-LLOYD, 1993).

The disadvantages of using molecular markers lie in the fact that they may be costly and time-consuming especially during the screening process (EDWARDS, 2000). Improvement in the screening technique can greatly reduce the costs.

Since the RAPD technique is based on DNA polymerase-mediated amplification and prone to physical and chemical variation, it is useful to optimize the components of the reaction cocktail (EDWARDS, 2000). This is also costly and time-consuming. The other major financial constraint of RAPD is the thermo-stable enzyme isolated from *Thermus aquaticus*, *Taq* polymerase (NEWBURY and FORD-LLOYD, 1993). Since RAPD is initiated from very small quantities of DNA, it is said to be prone to artefacts caused by contamination of the reaction mixture by foreign DNA (NEWBURY and FORD-LLOYD, 1993). The contaminants compete with the template DNA for primer binding sites. The other disadvantage of RAPD markers is less information supplied by the banding pattern (NEWBURY and FORD-LLOYD, 1993). More than 90 % of RAPD markers are inherited in a dominant fashion with loci defined by two alleles (band either present or absent) (EDWARDS, 2000). This does not allow one to discriminate between plants that are homozygous or heterozygous for a particular amplifiable sequence. In both cases a band of the same size is observed (NEWSBURY and FORD-LLOYD, 1993).

Although this technique has its own setbacks, it has however gained popularity and is currently being used in biotechnology for different purposes. It is mainly used for studying genetic diversity, constructing linkage maps, varietal fingerprinting and the identification of somatic hybrids (NEWBURY and FORD-LLOYD, 1993). In molecular ecology, RAPD markers are used exclusively for determining taxonomic identities, detecting inter-specific gene flow, assessing kinship relationships, analysing mixed genome samples and producing specific probes (HADRYS *et al.*, 1992).

The use of RAPD in tissue culture is well documented in the literature. RAPD was applied by PIOLA *et al.* (1999) as a tool to estimate genetic variation within and among *in vitro* propagated cedar clones. No genetic variation was detected within the microcutting clones but across the four intra-specific mother-plantlets. SHOYAMA *et al.* (1997) used RAPD to study the genetic fidelity of *Panax notoginseng* somaclones. The amplification products were homogenous which indicated that somatic embryogenesis could be used for clonal propagation of this plant.

The following experiments were designed to estimate the level of similarity of the micropropagated *Dierama luteoalbidum* plantlets using RAPD. Since the plantlets were derived from meristemoids (semi-organized callus tissue), the aim was to determine the effect meristemoid induction had on the clonal fidelity of the plantlets

5.2 GENOMIC DNA ISOLATION

a) Materials and Methods

Total genomic DNA was extracted from *in vitro* leaves in the culture jars using the modified Hexadecyl-trimethylammonium bromide (CTAB) (Janssen Chimica, Belgium) extraction protocol (RICHARDS, 1997). Clean latex gloves were worn at all times when working with DNA. Tips, Eppendorfs, mortars and pestles, spatulas were autoclaved prior to use.

Five hundred µl of the CTAB extraction buffer [2 % w/v CTAB, 100 mM Tris-Cl, (pH 8.0), 1.4 M NaCl, 20 mM EDTA, (pH 8.0)] were added to sterile 1.9 ml Eppendorf tubes already containing 3 % (w/v) PVPP and allowed to re-hydrate for 20-30 minutes in a 65 °C water bath. Frozen leaf material (100 mg), was ground to a fine powder in a mortar and pestle using liquid nitrogen. Prior to addition of the ground leaf powder to the Eppendorf tubes, 1 % (v/v) 2-mercaptoethanol was immediately added to the buffer in a fume-hood. The contents in the Eppendorf tubes were mixed thoroughly by inversion then incubated for 30 minutes in a 65 °C water bath. Thereafter, an equal volume of 24:1 (v/v) chloroform: isoamyl alcohol extraction was followed by centrifugation at 7500 *g* for five minutes in a bench-top centrifuge. The aqueous phase was transferred into new Eppendorf tubes and one-tenth of the volume of CTAB/NaCl solution (10 % w/v CTAB, 0.7 M NaCl) was added. The contents were mixed well by inversion and the chloroform: isoamyl alcohol extraction was repeated. The aqueous phase was transferred into new Eppendorf tubes and one volume of CTAB precipitation solution [1 % w/v CTAB; 50 mM Tris-Cl, (pH 8.0); 50 mM

EDTA, (pH 8.0)] added. The mixtures were incubated in a 65 °C water bath for 30 minutes. Centrifugation (500 *g*) for 5 minutes in a bench-top centrifuge followed and the pellet was resuspended in 500 µl high salt TE buffer [10 mM Tris-Cl, (pH 8.0); 0.1 mM EDTA, (pH 8.0); 1 M NaCl]. The DNA was precipitated by adding 0.6 of the volume of ice cold isopropanol and pelleted at 7500 *g* for 15 minutes. The supernatant was carefully removed and the pellet was washed once with 70 % (v/v) ethanol and immediately thereafter with 100 % ethanol. The pellet was air dried to remove excess alcohol and thereafter dissolved in 20-50 µl TE buffer [10 mM Tris-Cl, (pH 8.0), 1 mM EDTA, (pH 8.0)] for storage at –20 °C.

b) Results and Discussion

Good quality DNA was obtained from the fresh leaves as opposed to the frozen leaves (Figure 6). In preliminary DNA extractions two methods were used, the Urea-Extraction protocol developed by DELLAPORT *et al.* (1985) and CTAB DNA extraction developed by HILLS and VAN STADEN (2002). The Urea-Extraction method gave very poor results. The average purity of the extracted DNA was 61 % and the average DNA concentration was 0.111 $\mu g \mu l^{-1}$. Furthermore, the pellets turned brown during the extraction process. The CTAB extraction method gave better results. The DNA concentration improved to 0.850 $\mu g \mu l^{-1}$ while the purity was 78 % (Table 11). When the modified CTAB DNA extraction developed by RICHARDS (1997) was used, the purity and yield of the DNA improved (Table 11). The amount of DNA obtained was sufficient for RAPD experiments. *Dierama* possesses very tough leaves which proved problematic during grinding in liquid nitrogen. The success of any DNA isolation protocol lies in the grinding of the tissues for thorough penetration of the buffer and lysis of cells. Furthermore, other chemicals such as 2-mercaptoethanol and insoluble PVPP included in the extraction buffer, bind to contaminants such as phenolics, (that bind to nucleic acids), thereby alleviating the problem of phenolics (KIM *et al.*, 1997).

Table 11: The DNA concentration and purity of the *in vitro* grown leaves of *D. luteoalbidum* extracted using three different DNA extraction methods

DNA extraction method	Concentration ($\mu g \mu l^{-1}$)	Purity (%)
Urea	0.111	61.0
CTAB (HILLS and VAN STADEN, 2002)	0.850	78.0
CTAB (RICHARDS, 1997)	0.771	99.3

5.3 ANALYSIS OF ISOLATED DNA

a) Quantification

The DNA was quantified using a CARY 50 CONC UV-Visible Spectrophotometer (Varian Pty., Ltd., Australia). The amount of UV radiation absorbed by a solution is directly proportional to the amount of DNA in solution. At the 260 nm wavelength, an absorbance (A_{260}) reading of 1.0 corresponds to 50 µg of double stranded DNA per ml. The absorbance reading gives both the concentration and the purity of the DNA in solution.

To determine the absorbance, 4 µl of each sample were mixed with 0.996 ml of sterile TE buffer [10 mM Tris-Cl, (pH 8.0), 1 mM EDTA, (pH 8.0)] in a quartz cuvette, which were soaked in 1 % nitric acid. The absorbance was measured at 260, 280 and 320 nm. The DNA concentration was calculated as follows: (Corrected A_{260} x ΔE x diluting factor)/Total volume (ml), where corrected A_{260} = $A_{260} - A_{320}$ and ΔE = 40 $\mu g \mu l^{-1}$ for single stranded DNA or RNA, 50 $\mu g \mu l^{-1}$ for double stranded DNA and 20 $\mu g \mu l^{-1}$ for oligonucleotides. The purity of the DNA in solution was calculated using a ratio of A_{260} to A_{280} and converted to a percentage. Pure DNA has a ratio of 1.8 and was diluted to 10 ng for primer screening.

b) Visualization of results

A 1.5 % (w/v) Agarose gel (Hispanagar, Burgos, Spain) was prepared to observe the results. A 50 X Tris Acetate (TAE) buffer [0.04 M Tris-Cl, (pH 8.0); 0.002 M EDTA, (pH 8.0); 14.3 ml glacial acetic acid] was first prepared. Thereafter a 1 X TAE was prepared by taking 20 ml of the 50 X TAE buffer and making it up to a litre with distilled water. A 40 ml mini-gel was used in most cases and prepared by adding 0.6 g of the agarose to 40 ml of 1 X TAE buffer in a 100 ml volumetric flask. The agarose was dissolved by heating in a microwave and thereafter allowed to cool to about room temperature. Immediately the gel was stained with 2 µl of 0.5 $\mu g \mu l^{-1}$ ethidium bromide and finally allowed to set for 15-30 minutes. After setting the gel was placed in gel system tank and immediately covered with the 1 X TAE buffer. The DNA samples were loaded together with a loading buffer [50 mM NaOH, 1 mM EDTA, (pH 8.0), 2.5 % (v/v) glycerol and 0.025 % (w/v) Bromophenol blue] to carefully monitor the migration of the DNA. The electrophoresis was performed at 5 volts per cm for at least 3 hours before photographing the gel under UV light using UVItec Gel Documentation System Doc-008 TFT.

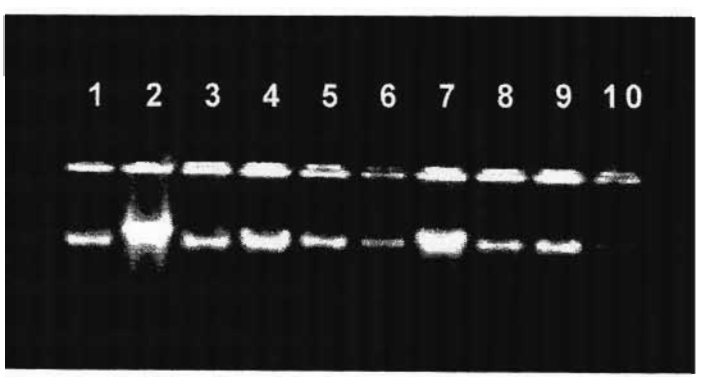

Figure 6: Analysis of *D. luteoalbidum* genomic DNA isolated from fresh and frozen leaf samples

DNA samples in lanes 2, 4, 5, 7 and 9 were isolated from fresh leaves and samples in lanes 1, 3, 6, 8 and 10 were isolated from frozen leaf material

5.4 RAPD

a) Materials and Methods

The reaction mixture (25 µl) for primer screening contained: 60 ng template DNA, 1 x PCR buffer (Roche, Germany), 0.5 µM 10-mer primer (Operon Technologies Inc., CA, USA), 0.2 mM of each dNTP (Roche, Germany), 1.5 units of *Taq* DNA polymerase (Roche, Germany) and sterile High Power Liquid Chromatography HPLC water (Labscan, Dublin) to make up to 25 µl. The negative control mixture consisted of all reagents except the template DNA. Each reaction mixture was carefully overlaid with 30 µl of mineral oil to prevent evaporation. The amplification was performed in an automated Hybaid Thermal Reactor (Hybaid Limited, UK) programmed for one cycle of 94 °C for 1 minutes, 36 °C for 20 seconds and 72 °C for 2 minutes; followed by 45 cycles of 94 °C for 10 seconds, 36 °C for 20 seconds and 72 °C for 2 minutes; and the final extension cycle of 72 °C for 5 minutes and 35 °C for 1 minute. A total number of 24 primers were used for their ability to generate RAPD markers in the screening process. For visualization of results, the electrophoresis gel was prepared and run as outlined in Section 5.2 b (ii).

From the 24 primers used in the screening process (Figure 7), six primers namely: OPB-01, OPB-11, OPB-18, OPB-17, OPB-10 and OPC-01 were chosen for further analyses (Table 12). Primer selection was based on the intensity and number of bands produced by each primer following amplification. Primers that produced five or more clearly visible bands were the ones selected.

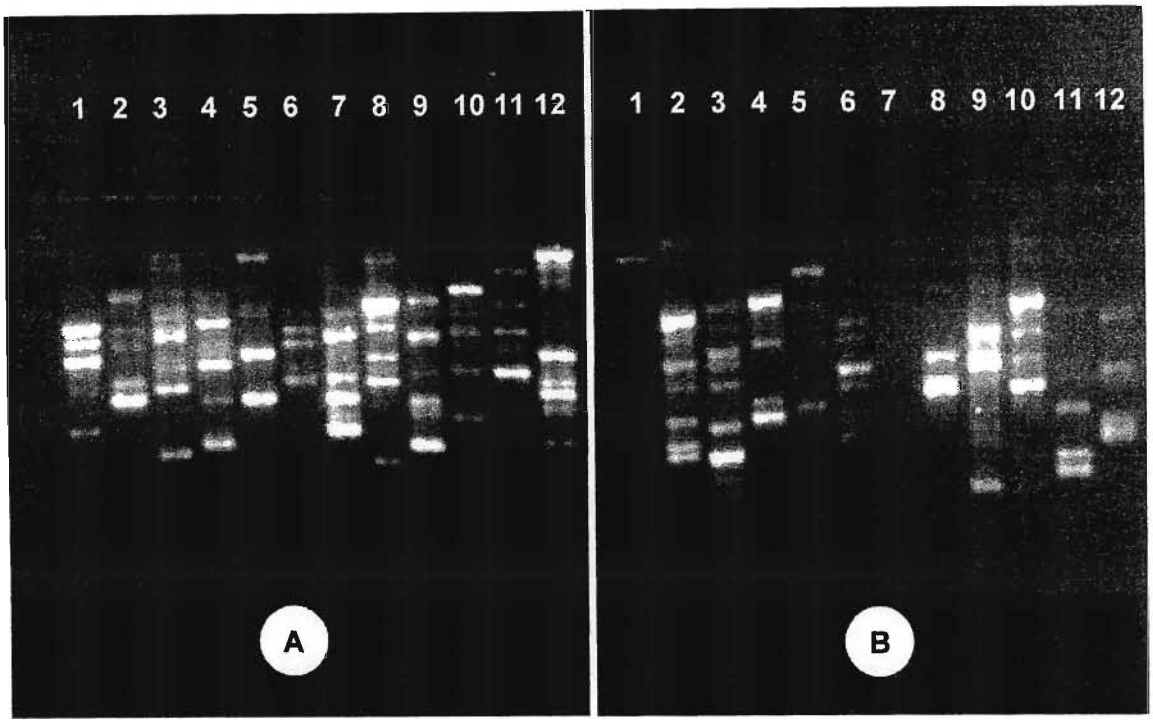

Figure 7: The RAPD products of the 24 randomly selected primers in the primer screening process

Lane 1) OPA-11; 2) OPA-15; 3) **OPB-01**; 4) OPB-04; 5) OPB-05; 6) OPB-09; 7) **OPB-10**; 8) **OPB-11**; 9) OPB-12; 10) OPB-13; 11) OPB-14; 12) OPB-15 (Gel A)

Lane 1) OPB-16; 2) **OPB-17**; 3) **OPB-18**; 4) OPB-19; 5) OPB-20; 6) **OPC-01**; 7) OPC-03; 8) OPC-04; 9) OPC-05; 10) OPC-06; 11) OPC-07; 12) OPC-08 (Gel B)

Table 12: The six selected primers of arbitrary sequences and the number of bands produced by each primer in the screening process

Primer	Sequence	Number of bands
OPB-01	5'-GTTTCGCTCC-3'	6
OPB-11	5'-GTAGACCCGT-3'	9
OPB-18	5'-CCACAGCAGT-3'	9
OPB-17	5'-AGGGAACGAG-3'	8
OPB-10	5'-CTGCTGGGAC-3'	5
OPC-01	5'-TTCGAGCCAG-3'	10

b) Results and Discussion

RAPD was used to check for genetic uniformity in 22 randomly selected DNA samples using the six selected primers. The RAPD bands separated by electrophoresis were scored manually as discreet variables, using 1 to indicate presence and 0 to indicate absence of a band. Only intensely stained bands were scored. Thereafter the Mean Similarity Matrices for each primer were calculated using a Single Linkage Hierarchical Cluster Analysis in Genstat™ 5 release 4.1 (1993).

The results presented here are for 3 out of the 6 selected primers namely: OPB-11, OPB-18 and OPC-01. The banding patterns of all the micropropagated plants looked similar (Figure 8A lane 1 to 23). The sample in lane 17 was from leaf material of plantlets that were multiplied on solid rather than liquid media. The sample in lane 25 is *D. luteoalbidum* collected from the wild and grown in the greenhouse. There were a total of 282 bands amplified by primer OPB-11. Two of the bands (2.8 and 0.9 kb in size) were present in all the micropropagated plants plus the wild plant. While most of the bands were common to all the micropropagated plants, there was one extra band of 1.0 kb in the liquid-cultured sample in lane 18. This sample lacked the common bands of sizes 2.7 and 2.6 kb. These bands were also absent in the samples in lane 2, 11 and 25 (wild plant). This primer yielded a total of 162 bands and an average of 8.7 polymorphic bands (Table 13).

Primer OPB-18 revealed a total of 225 bands ranging from 2.8 to 0.5 kb. The banding patterns of all micropropagated plants also looked similar (Figure 8B lane 1 to 23). There were at least five common bands in the micropropagated plants and garden plant. The sizes of the common bands were 1.7, 1.6, 1.2, 0.7 and 0.5 kb respectively. While most bands were common in the micropropagated plants, there was an extra band (1.8 kb) in lane 17, representing one sample multiplied through solid media. This sample also lacked the band common to all the micropropagated plants of approximately 0.9 kb. The plant cultured through liquid media in lane 18, lacked the band common in all the micropropagated

plants of size 3.1 kb. The primer yielded a total of 105 bands and an average of 4.3 polymorphic bands (Table 13).

Primer OPC-01 yielded a total of 237 bands ranging in size from 5.1 to 0.2 kb. There were only two bands that were common in all the micropropagated plants plus the garden plant, of sizes 1.8 and 0.6 kb respectively. The majority of the bands were common in all the micropropagated plants despite an extra band of 3.0 kb in lanes 11, 19, 22, 23 and 24. Overall the primer yielded a total of 140 bands and an average of 5.8 polymorphic bands (Table 13).

Table 13: The level of similarity of micropropagated *D. luteoalbidum* plants and the average number of bands as revealed by each primer

Primer	Mean Similarity (%)	Polymorphic bands	Monomorphic bands
OPB-11	87.5	6.7	5.0
OPB-18	81.5	4.3	5.0
OPC-01	82.8	5.8	4.0

This study clearly demonstrated how RAPD can be used in the estimation of the clonal fidelity of micropropagated plants. It showed how short 10-mer primers of arbitrary nucleotide sequence may be used to reproducibly amplify segments of DNA from different sources. If the banding pattern of individual genomes were similar, then the level of similarity of the samples would also be expected to be high.

The level of similarity in the micropropagated plants was more than 80 % and hence most of the bands were common to all of the plants. This implies that the micropropagation protocol yielded plantlets that are at least 80 % genetically similar. There were very few polymorphic bands detected by the primers that were used. The polymorphism in amplification products could have resulted from changes in either the sequence of the primer binding site or changes which altered the size or prevented the successful amplification of the target DNA.

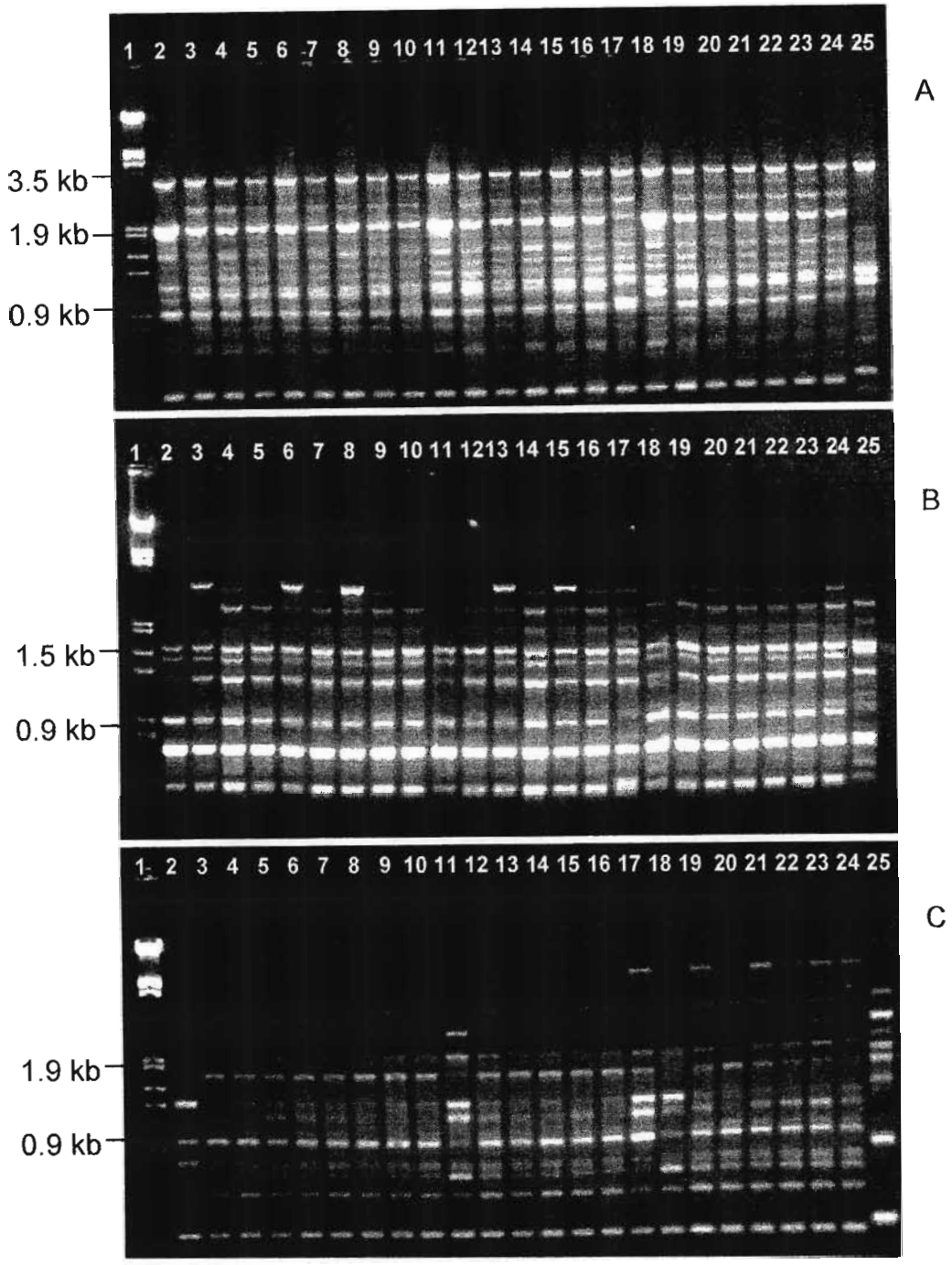

Figure 8: The RAPD products of *D. luteoalbidum* DNA isolated from *in vitro* leaves amplified with primers OPB-11 (Gel A), OPB-18 (Gel B) and OPC-01 (Gel C)

Lane 1) MWM III; lane 2-10,11-16 and 18-24 plants derived from three different meristemoid lines; lane 17) plant multiplied through solid media; and, lane 25) *D. luteoalbidum* collected from the wild

The changes in the target DNA can be attributed to insertions, deletions or inversions in the nucleotide sequence (RANI *et al.,* 1995). According to WILLIAMS *et al.* (1990) RAPD can detect just a single base change in genomic DNA by introducing a mismatch in the primer-DNA duplex. However, not all amplification products are a result of perfect pairing between primer and template DNA.

The level of similarity (genetic uniformity) in micropropagated plants seems to be affected by the type of tissue the plantlets are derived from. For example, somatic embryos from callus derived from young flower buds were found to be genetically uniform in *Panax notoginseng* (SHOYAMA *et al.,* 1997). The culture conditions also play a role in the genetic uniformity of micropropagated plants. In *Cyclamen persicum* Mill somatic embryos were induced from callus derived from immature ovaries. The callus was induced by adding 2,4-D to the growth medium. Although there was genetic uniformity in the samples, at high concentrations of 2,4-D the uniformity was lost (LAURA *et al.,* 2003).

In this study, plantlets were derived from meristemoids formed during the total immersion of shoots in liquid medium. Meristemoids are callus-like structures made up of highly specialised and differentiated cells (GEORGE, 1993). In contrast, callus is a mass of proliferating cells that are undifferentiated and unspecialised. This explains the reason for somaclonal variation reports in some micropropagated plants. For clonal propagation purposes, it is advisable to use organized meristems, as they are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions (RANI *et al.*, 1995). There are reports of producing genetically uniform plantlets from axillary buds, although the inclusion of plant growth regulators is sometimes known to induce somaclonal variation (PIOLA *et al.,* 1999).

In conclusion, meristemoids with organized meristems, yielded little genetic variation using 3 selected primers. This suggests that meristemoids can be used in the clonal propagation of *Dierama* species for conservation purposes. Other workers used plant growth regulators such as anti-gibberellins for initiation of meristemoids (ZIV, 1989). In this study, meristemoids were initiated from a low

BA supplemented liquid MS medium. Plant growth regulators are known to indirectly affect somaclonal variation by increasing the multiplication rate (BAIRU, 2004). It may, therefore, be necessary to investigate the effect of hormones (particularly at high concentrations) on the genetic fidelity of the micropropagated plants.

CHAPTER 6: STORAGE, VIABILITY AND GERMINATION OF THE SEEDS

6.1 INTRODUCTION

Angiosperm seeds result from the fusion of the male pollen with the female egg nucleus and play a critical role in the life cycle of these higher plants (BEWLEY and BLACK, 1985). The mature seed plays an important role in dispersal, survival and establishment of the germinating seedling. Before germination can occur, the seedling has to adapt to the environment and this process differs from species to species. The general trend is the uptake of water by the seed, which is coupled with the hydrolytic activity of enzymes that break down the stored reserves to provide food for the growing embryo (BEWLEY and BLACK, 1985). Germination is switched on once the seed is imbibed and ends when the radicle emerges and elongates (BEWLEY and BLACK, 1985).

Seed banks are available all over the world for the storage of endangered or rare plant germplasm. The primary aim of seed banking is to provide a reserve of propagules for conservation purposes. There are two types of conservation strategies used for the increased survival of plants in the wild. *Ex situ* conservation involves storing and growing the plants in the laboratory using *in vitro* techniques or in the garden while *in situ* conservation deals with the protection of the plant's habitat. There needs to be a balance between the two strategies for successful conservation. Storing seeds in a seed bank is inexpensive, requires a small space and allows for the conservation of rare and endangered plants without affecting the genetic diversity in the wild.

Medium-term storage aims to reduce growth of the *in vitro* material by modifying environmental conditions (temperature and light). Long-term storage involves cryopreservation, whereby all metabolic processes are inhibited at ultra low temperature. *In vitro* produced plantlets can be stored in this way (ENGELMANN, 1998). Cryopreservation of seeds has also gained popularity and was shown not

to interfere with the viability of the seeds (BEWLEY and BLACK, 1985). Freeze-drying is effective in some seeds and very harmful in others (BEWLEY and BLACK, 1985).

For storing germplasm seeds are carefully sampled and their genetic composition preserved using available procedures (TOWILL, 1988). It is well known that cool temperature, low moisture content and low oxygen tension help maintain the viability of seeds during storage (ROBERTS, 1973). This is true for a large number of species which produce seeds that are termed orthodox (ENGELMANN, 1998). In contrast, there are species that are high in moisture and cannot be stored at low temperatures; these seeds are termed recalcitrant (CORBINEAU and CÔME, 1988). These seeds cannot be stored in normal seed banks. The development of alternative methods for storage of recalcitrant seeds is still underway.

While orthodox seeds remain viable when stored for some years, recalcitrant seeds loose their viability within a few weeks to a few months (BEWLEY and BLACK, 1985). Recalcitrant seeds must maintain relatively high moisture content in order to remain viable (CORBINEAU and CÔME, 1988). Orthodox seeds require low moisture content for storage purposes. A slight increase in the moisture content makes the seeds susceptible to insect or microbial attack. A significant increase in moisture content is not suitable for storage purposes as it allows germination to occur (BEWLEY and BLACK, 1985).

Temperature and relative humidity (RH) also have an effect on the viability of the seeds during storage. Recalcitrant seeds are predominantly seeds from tropical or subtropical trees and shrubs (CORBINEAU and CÔME, 1988), in other words from relatively warm climates. At low temperatures these seeds lose their viability unless the high moisture content is maintained (CORBINEAU and CÔME, 1988). In contrast, orthodox seeds can be stored at low temperatures in moisture-proof containers where the RH is kept low (BEWLEY and BLACK, 1985).

There are problems associated with the conservation of seeds: a) some species produce orthodox seeds that are highly heterozygous and these are of limited

interest for the conservation of particular genotypes; b) some species are only propagated vegetatively since they do not produce seeds; and, c) some species produce recalcitrant seeds that cannot be stored for longer periods (ENGELMANN, 1998).

Dierama seeds require a lot of water and readily germinate when sown (HILLIARD and BURTT, 1991). However there is no information available on the effect of seed age on viability of these seeds. As 18-month old *D. luteoalbidum* seeds had shown a poor germination response *in vitro* (CHAPTER 2), the aim of the following experiments was to understand the relationship between short-term storage and the viability of *Dierama* seeds. The poor germination response could have been due to dormancy or loss of viability of the seeds. Preliminary experiments were performed to break dormancy (data not shown). The seeds were chemically scarified with 70 % hydrogen peroxide and the seed coat was disrupted with sand paper. Germination, however, did not improve and mechanical scarification resulted in no germination. The poor germination response was attributed to a loss in seed viability as the seeds aged. These experiments were also designed to investigate the effect of temperature on the germination of the seeds. Temperature is critical in seed germination since it may be involved in removing or inducing dormancy. Secondly, temperatures at which seeds are incubated determine their rate of germination (BEWLEY and BLACK, 1982; cited by HILLS, 2003).

6.2 GENERAL MATERIALS AND METHODS

D. igneum seeds were purchased from the Croft Wild Bulb Nursery (Eastern Cape, South Africa) in August 2002. They were stored in a brown envelope at room temperature prior to use.

6.3 EFFECT OF TEMPERATURE ON SEED GERMINATION

a) Materials and Methods

Fifteen months old *D. igneum* seeds were surface sterilized in plastic sieves. They were dipped in 0.1 % $HgCl_2$ for 2 minutes then washed thoroughly in tap water and finally in distilled water. Thereafter, the seeds were germinated at temperatures of 10, 20, 25, 30, 35 and 40 °C on 90 mm Whatman number 1 moist filter paper in plastic Petri dishes. The chambers were given 16 hours light: 8 hours dark regime. Unfortunately the light intensity was not consistent for all the chambers (Table 14). The water uptake of the seeds prior to germination at 25 °C was carefully monitored. Germination was considered complete once the protruding radicles were at least 2 mm long. Ten seeds were used per treatment and each treatment consisted of three replicates. The experiment was allowed to proceed for 14 days.

Table 14: The light intensity in the growth chambers at each temperature to test the effect of temperature on seed germination

Temperature (°C)	Light irradiance (µmol m^{-2} s^{-1})
0	23.2
10	38.6
20	21.9
25	27.8
30	64.4
35	45.5
40	47.9

b) Results and Discussion

Temperature does have an effect on the germination of the seeds. Germination increased with an increase in temperature. A good germination response was between 25 and 30 °C with 100 % germination at 25 °C. The optimum temperature for germination was 25.84 °C (as calculated below table 15).

Increasing the temperature to 35 °C resulted in only 10 % of the seeds germinating. At low temperatures (10 °C) and at a higher temperature of 40 °C no germination was recorded (Table 15). However, these seeds immediately germinated when the temperature was shifted to 25 °C. This thermo-dormancy (inhibition) needs further investigation. The mean germination time was calculated (as below table 15). Seeds germinated within 10 days when kept at 25 °C. There was a detrimental effect of temperature at 35 °C, as seeds took 11 days to reach 10 % germination (Table 15). The seeds had an average moisture content of 15.1 %.

Table 15: The effect of constant temperature on the germination of *D. igneum* seeds

Temperature (°C)	Germination (%)	Mean germination time (days)
10	0.00 c	0.00
20	70.0 b	12.4
25	100 a	10.4
30	96.7 a	10.6
35	10.0 c	11.0
40	0.00 c	0.00

LSD (5 %) = 11.09

1) Mean germination time = $\sum$ (n x d)/N, where **n** is the number of seeds that germinated after each period of incubation in days **d** and **N** is the total number of seeds germinated during the test period.
2) Optimum temperature = $\sum$ tp/ $\sum$p, where **t** is the temperature and **p** the percent germination.
3) Moisture content = [(Fresh weight – Dry weight) / Fresh weight] x 100

There was no significant difference in the germination response of seeds germinated at 25 and 30 °C. Each species has a range of temperatures in which germination may occur (HILLS, 2003). The optimum germination temperature, defined as the temperature at which germination occurs in the shortest possible period of time, was at 25 °C, a relatively warm temperature. This implies that

germination in the field will be restricted to warmer months. This is not surprising as *Dierama* is found only in areas that receive a summer rainfall (HILLIARD and BURTT, 1991). This indicates that the seeds wait for warm temperatures 25-30 °C and rain before they germinate. This further explains the reason why there was no germination at 10 and 40 °C and why germination resumed once the temperature was shifted to 25 °C. In contrast, *Watsonia fourcadei* seeds were found to germinate optimally at 10 and 15 °C (ESTERHUIZEN *et al.*, 1986). *Watsonia,* just like *Gladiolus,* is found in areas that receive a winter rainfall (HILLIARD and BURTT, 1991). Therefore, the difference in germination response in *Dierama* and *Watsonia* can be attributed to different ecological habits. The inhibition of germination at low and high temperatures should be investigated further in other *Dierama* species found in different habitats, to determine the germination requirements in these species.

To determine the amount of water that needs to be absorbed by the seed before germination can occur at 25 °C, the imbibition rate was carefully monitored. It was found that there was a steady increase in the first 12 hours. Thereafter, the water uptake doubled every 12 hours until 96 hours, which was when germination started. This shows that for germination to occur at 25 °C, the seeds need to attain 60% imbibition (Figure 9).

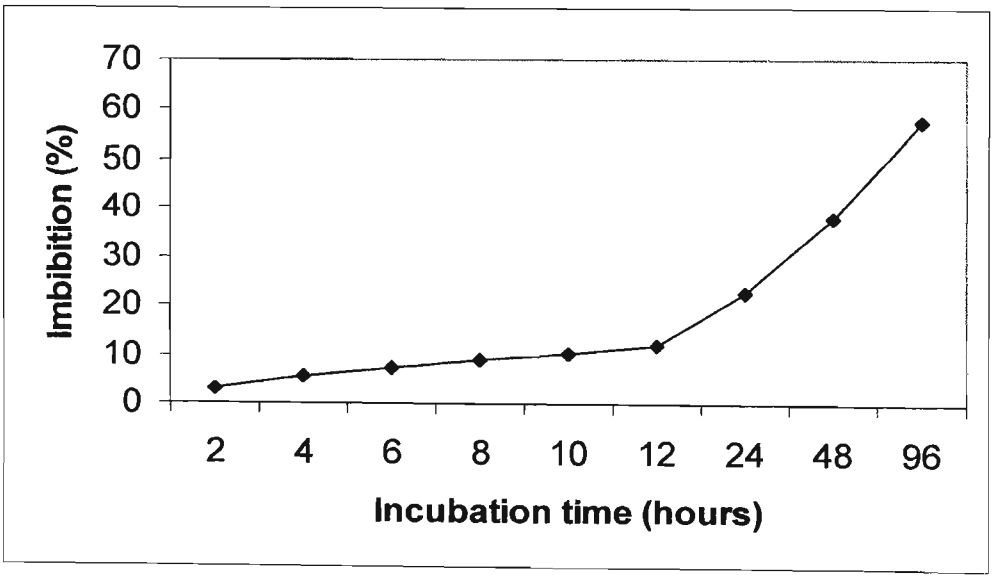

Figure 9: The imbibition rate of *D. igneum* seeds up to 96 hours at room temperature

Seed coat permeability is one of the factors that affect the rate of imbibition in germinating seeds (McDONALD and COPELAND, 1989). Seeds clearly started absorbing water during the early stages of incubation (Figure 9). However, after 12 hours the amount of water absorbed by the seed increased significantly. At this point the seed coat was permeable enough to allow the rapid influx of water. The pattern of water uptake by germinating seeds indicates that there are three phases (BEWLEY and BLACK, 1985): Phase 1 is the rapid water uptake by the seed; phase 2 is the lag phase where major metabolic events take place in preparation for radicle emergence; and, phase 3 is the elongation of the radicle coupled with a higher water uptake by the seed from the soil (BEWLEY and BLACK, 1985). The graph (Figure 9) represents the first phase in water uptake by the germinating seeds. If the experiment was allowed to proceed beyond 96 hours, the lag phase would be represented in the graph.

6.4 EFFECT OF STORAGE ON SEED VIABILITY

a) Materials and Methods

D. igneum seeds were stored for six months at 0, 10, 20 and 30 °C in 70 mm plastic Petri dishes, either in 16 hours light: 8 hours dark (Table 14) or in complete darkness. For the dark treatment, the Petri dishes were wrapped with aluminium foil. Twenty seeds were used per treatment and each treatment consisted of three replicates. Following the storage period the seeds were tested for viability and germinated.

i) Viability testing of the seeds

The seeds were imbibed for 48 hours and thereafter were soaked in 0.1 % 2,3,5-triphenyl tetrazolium chloride (TTC) for 24 hours in complete darkness at room temperature. The viable seeds were scored as those that turned reddish-purple in colour.

b) Results and Discussion

Using 0.1 % TTC only 50 % of the freshly collected seeds were viable. No major differences were observed in the seeds stored in the light or dark. After 2 months, the viability of the seeds was lower than 25 %. After 4 months, the percentage viability remained unchanged in the seeds stored in the light and was more than 30 % in the seeds stored in the dark. After 6 months, approximately 30 % of the seeds stored in both the light and dark were viable (Table 16).

Table 16: The effect of storage time on the viability of *D. igneum* seeds as tested using 0.1 % TTC

Time (months)	Viability (%)	
	light	dark
0 (control)	50	-
2	21.1	20.0
4	21.1	33.7
6	32.2	28.3

Storing seeds at lower temperatures yielded slightly more viable seeds (Table 17). Only 30 % of the seeds stored at 0 °C and 10 °C were viable. Storing seeds at 20 and 30 °C yielded viability percentages less than 30 %. Seeds stored in the dark were more viable than seeds stored in the light (Table 17).

Table 17: The effect of storage temperature on the viability of *D. igneum* seeds using 0.1 % TTC

Temperature (°C)	Viability (%)	
	light	dark
0	31.2	26.2
10	31.2	33.3
20	21.2	25.0
30	21.2	25.0

Seed viability testing offers an advantage over germination testing since it identifies dormant seeds as viable which would otherwise be regarded as dead in the germination test (McDONALD and COPELAND, 1989). For this purpose a colourless stain of TTC was used. It is taken up by the seed and then hydrogenated. Only the viable seeds produce triphenyl formazan, a red to purple colour indicator (ANONYMOUS, 1999).

When using 0.1 % TTC, the percentage viability of the fresh (not stored) seeds was 50 %. It was expected that as time progressed the viability of the seeds would decrease. It was also expected that storage of the seeds at higher temperatures would also lower the viability. When 1.0 % TTC was used on 15 month-old seeds the viability was 87 % which more or less correlated with the germination response (100 % at 25 °C). With 0.1 % TTC, the viability of freshly collected seeds was 50 % while the average germination at 25 °C was 90 %. Thereafter, when seeds were stored for up to 6 months, the viability was less than 35 % while the average germination was more than 90 %. Although imbibing the seeds to allow for the penetration of the stain plays a large role in the success of the TTC viability test, it did not pose a problem in this case. *Dierama* seeds are small and do not possess a very hard seed coat (HILLIARD and BURTT, 1991). Therefore imbibing the seeds for 48 hours was sufficient to weaken the seed coat. In very hard seed coated seeds, the embryo is normally excised or the seed is cut in half prior to adding the stain (ANONYMOUS, 1999). It is therefore suggested that at least 1.0 % TTC should be used for viability testing of *Dierama* seeds in the future. The suggested concentration of TTC for viability testing ranges from 0.1 to 1.0 % (ANONYMOUS, 1999).

b) Germination of the seeds

i) Materials and Methods

The seeds were germinated on moist 70 mm Whatman number 1 filter paper in plastic Petri dishes under the 16 hours light: 8 hours dark regime at ± 25 °C. Light was supplied by 'cool white' fluorescent tubes at an irradiation of 43 µmol

m^{-2} s^{-1}. Germination was considered complete once the protruding radicles were at least 2 mm long. No germination was conducted in the dark. The germination experiment was allowed to proceed for a period of 20 days.

ii) Results and Discussion

Fresh seeds start germinating on the second day and reach maximum germination between days 10 to 20 (Figures 10-12). There was a delayed germination response in seeds stored for two months at 0 and 20 °C (Figure 10). Seeds from other treatments reached 50 % germination on the sixth day and reached maximum germination between days 10 and 20. There was a delayed germination response in seeds stored for four months at 20 °C (Figure 11). Seeds from other treatments reached 50 % germination on the sixth day and maximum germination between days 12 and 20. There was a delayed germination response in all seeds after six months (Figure 12). It took most seeds 14 days to reach 50 % germination and none of the seeds reached 100 % germination (Figure 12).

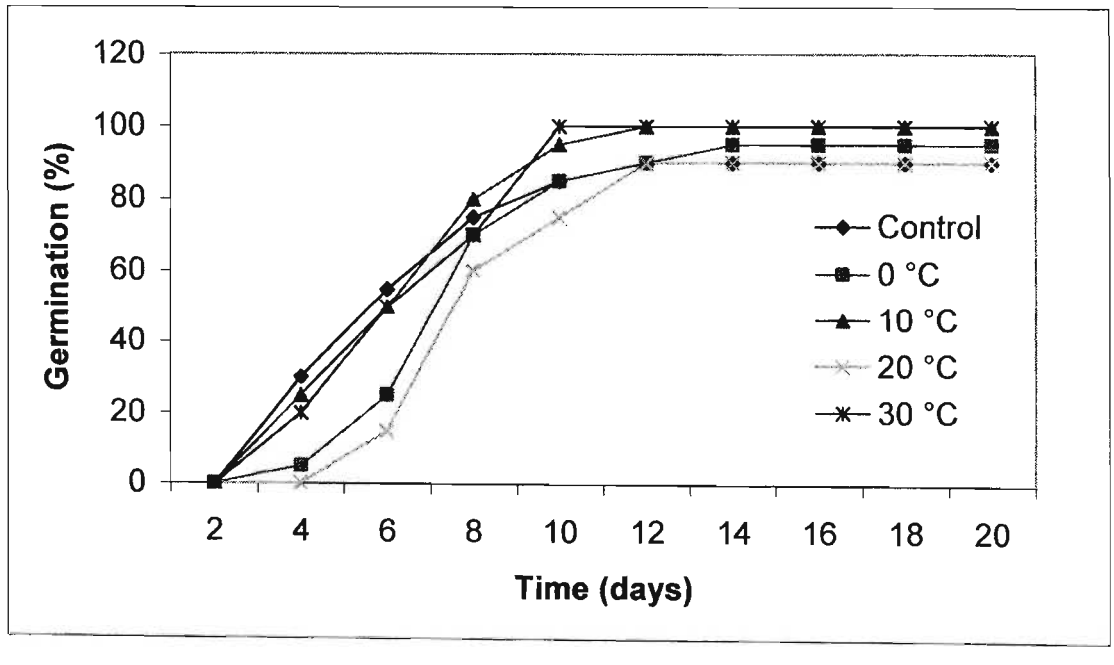

Figure 10: The germination rate of *D. igneum* seeds stored for two months at 0, 10, 20 and 30 °C

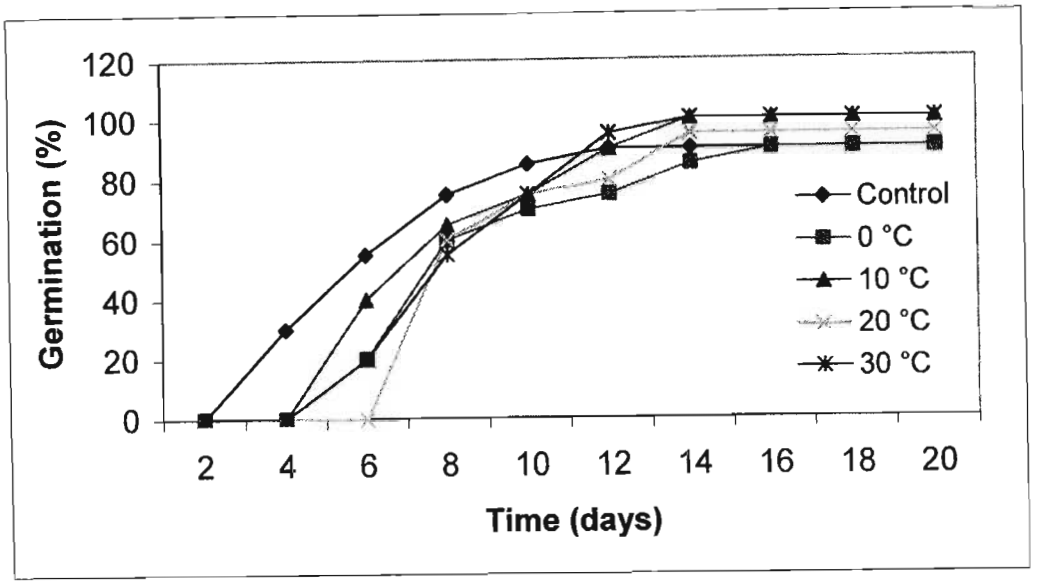

Figure 11: The germination rate of *D. igneum* seeds stored for four months at 0, 10, 20 and 30 °C

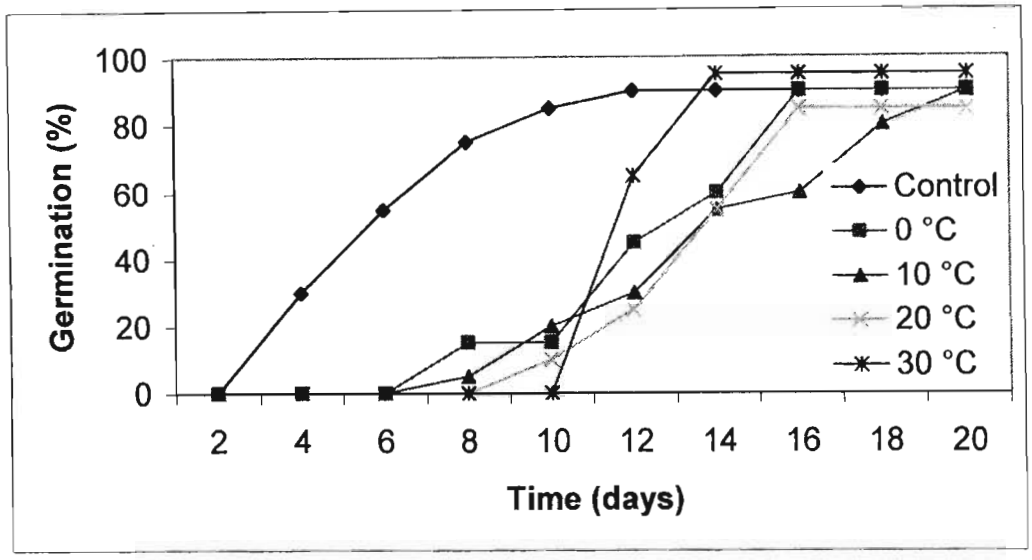

Figure 12: The germination rate of *D. igneum* seeds stored for six months at 0, 10, 20 and 30 °C

Storage time affected the germination of the seeds. The germination response of the seeds stored for two and four months remained unchanged but decreased after six months. In all cases, a greater number of seeds germinated in the dark treatment compared to those stored in the light (Figure 13).

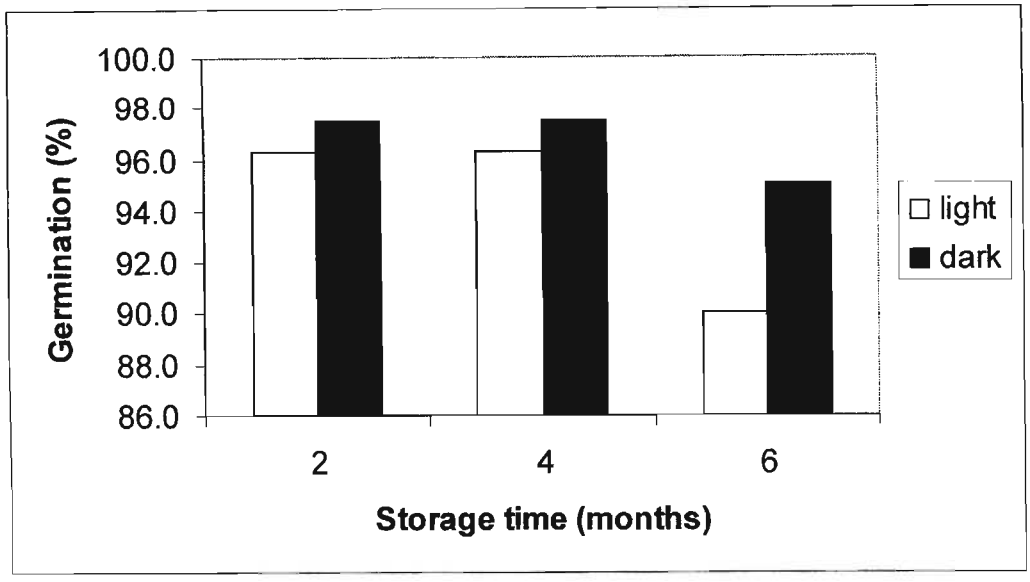

Figure 13: The effect of storage time on the germination of *D. igneum* seeds

Dierama seeds have an average moisture content of 15 %, which classifies them as orthodox. Theoretically, this indicates that they could be stored for longer periods without losing viability. The slight decrease in germinability after six months can be attributed to the aging of the seeds. Furthermore, the germination rate reduced when seeds were stored for longer periods (Figures 10-12). Temperature affects both the capacity to germinate and the germination rate (BLACK and BEWLEY, 1985). The mechanism by which germination is delayed as a result of storage is not very clear. There are some biochemical factors that lead to a loss of viability during storage namely: a) damages to DNA and cellular membranes; and, b) lack of respiration and ATP synthesis (BLACK and BEWLEY, 1985). When seeds are stored, they should be stored in well aerated areas that are also low in moisture. Unfortunately, there were very few studies with which these results could be compared.

Although there was a decrease in germination over time, it was not significant. There was again no significant effect of temperature and light during storage on the germination of the seeds. Seeds stored at temperatures of between 20 and 30 °C germinated more readily than seeds stored at lower temperatures (Figure 14).

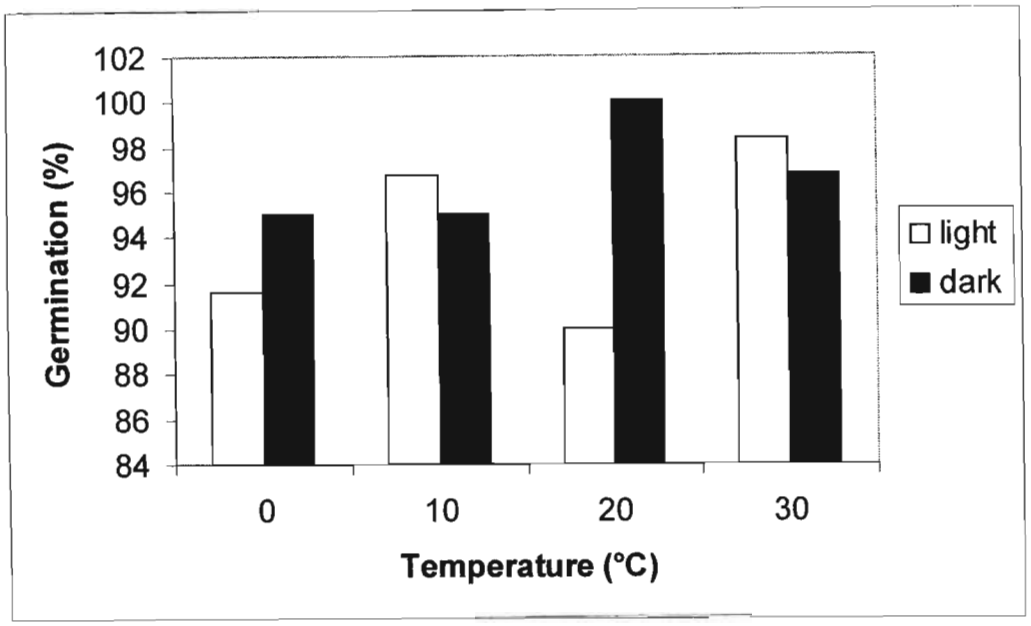

Figure 14: The effect of temperature and light during storage on the germination of *D. igneum* seeds

The germination temperature was kept constant and the only fluctuations were in the storing temperatures. Although seeds are generally stored at low temperature and moisture in order to retain viability, it appears that *Dierama* seeds can be stored for up to six months in temperatures ranging from 0 to 30 °C without losing viability.

In conclusion, the results obtained add some value towards the *ex situ* conservation of endangered *Dierama* species. Firstly, *Dierama* seeds have a moisture content of 15 %, which implies that they are orthodox. This means that the seeds can be stored for longer periods without losing viability. Unfortunately, this study did not determine the time it takes for the seeds to lose viability. It also did not answer the question why so few *D. luteoalbidum* seeds germinated *in vitro*. *Dierama* seeds can be stored for six months, at temperatures ranging from 0 to 30 °C, without losing germinability. The seeds incubated at 25 °C will take only 10 days to reach 100 % germination.

CHAPTER 7: GENERAL CONCLUSIONS

Dierama luteoalbidum and *D. erectum* are endemic to the KwaZulu-Natal Midlands and are listed among the top five endangered *Dierama* species in the province. Although *Dierama* propagates vegetatively via corms and seeds, this process is slow. In order to provide an *ex situ* reserve of propagules, tissue culture was successfully employed in this study.

The decontamination of corms was carried out using various disinfecting treatments all of which made use of JIK (3.5 % NaOCl). The decontamination procedure used was not very successful as all the decontaminated corms were later infected with endogenous micro-organisms. Even hot-water did not alleviate the contamination problem. It was suggested that more powerful disinfectants such as $HgCl_2$ could be utilised to eliminate the soil-borne micro-organisms.

The suitability of seeds as possible explants was investigated. *D. luteoalbidum* and *D. erectum* seeds were successfully decontaminated with 70 % ethanol and 3.5 % NaOCl (JIK) and germinated *in vitro* on MS medium. *D. erectum* seeds were germinated on MS medium supplemented with 0.5 mgL^{-1} BA. It was found that the seeds germinated better on MS medium devoid of BA. It was also found that the low germination response in *D. luteoalbidum* was a consequence of the age of the seeds. For multiplication purposes, hypocotyls were placed onto different concentrations of BA and NAA.The hypocotyls formed multiple shoots in MS medium supplemented with 0.5 mgL^{-1} BA after six weeks. Increasing this concentration and including NAA in the medium resulted in callus formation. For bulking-up of the plant material, the shoots were cultured in liquid MS medium supplemented with 0.5 mgL^{-1} BA. After six to eight weeks in culture, the shoots gave rise to meristemoids which elongated to form shoots once transferred to the same medium solidified with 2 gL^{-1} Gelrite. The progressive growth in liquid media was found to be greater than that on solid media. For proliferation purposes, *Dierama* shoots can be transferred to a liquid-shake MS medium containing 0.5 mgL^{-1} BA. The meristemoids reduce the chances of hyperhydricity and elongate forming shoots when transferred to a solidified medium.

Dierama shoots formed roots on MS medium supplemented with 6-8 % sucrose and 5 gL^{-1} activated charcoal after six weeks. The higher the sucrose concentration, the higher the number of roots. In the presence of charcoal, only root length improved. Shoots left undisturbed on this medium formed corms after six months. More corms were formed on a medium containing 8 % sucrose. The addition of 1-10 mgL^{-1} paclobutrazol reduced the corm induction period to three months. More corms were produced at 10 mgL^{-1} paclobutrazol. Paclobutrazol inhibited the formation of roots while it had no effect on the elongation of leaves. Formation of corms *in vitro* offers the advantage of increasing the survival of plants in the greenhouse. The survival of the rooted plantlets was determined on two different soil types. Plants survived better on the peat: compost: bark (1:1:1) v/v soil mixture than the soil: sand: vermiculite potting mixture (1:1:1) v/v. The plants formed larger corms after six months when treated with 1 % Kelpak. Formation of corms *in vitro* also has an advantage since the corms produced can be further sub-divided for multiplication purposes.

The genetic fidelity of the micropropagated plants derived from meristemoid clusters was determined using RAPD analysis. DNA was extracted from *in vitro* leaves using a modified CTAB DNA extraction method. Extracting DNA from fresh leaves yielded more DNA than extracting DNA from frozen leaves. The RAPD primers used namely OPB-11, OPB-18 and OPC-01, yielded little genetic variation in the micropropagated plants. The plants derived from meristemoids were compared to a plant derived from solid medium. The micropropagated plants shared most of the bands and hence their level of similarity was more than 80 %. This suggested that meristemoids (organized callus) can be used in the clonal propagation of *Dierama* species for conservation purposes.

There was a poor germination response of 18-month old *D. luteoalbidum* seeds *in vitro* despite using germination enhancement techniques. After preliminary viability tests with TTC, the viability was found to be very low. This was thought to be attributed to the age of the seeds. Freshly collected *D. igneum* seeds were used to study the effect of storage on seed viability. It was found that these seeds could be stored for up to six months in temperatures ranging from 0 to 30

°C without losing viability although the concentration of the stain used affected the results. The longer the storage time, the longer it took the seeds to start germinating. These seeds were found to have an average moisture content of 15 %, meaning they can be classified as orthodox. This however does not explain why *D. luteoalbidum* seeds had a poor germination response. Seeds germinated at 25 °C, took only 10 days to reach 100 % germination. There was no germination recorded at 10 and 40 °C, but only when the temperature was shifted to 25 °C. It was suggested that perhaps the germination of these seeds was thermo-inhibited at temperatures much lower or much higher than 25 °C. Such speculation can be investigated further in the future.

As a final point, the research conducted in this investigation has clearly illustrated the benefits of using the plant tissue culture system for the conservation of endangered *Dierama* species.

LITERATURE CITED

ALLAN, E. 1991. Plant cell culture. In: Stafford, A. and Warren, G. (Eds.) Plant Cell and Tissue Culture. Open University Press, London. ISBN 0-355-15832-4

ANONYMOUS. 1999. International rules for seed testing. *Seed Science and Technology (Supplement)* **27**: 33-35.

BACH, A. 1987. The capability of *in vitro* regeneration of various cultivars of *Freesia hybrida*. *Acta Horticulturae* **212**: 715-718.

BAIRU, M.W. 2004. Plant growth regulators and somaclonal variation in Cavendish banana (*Musa* AAA cv. *Zelig*). (M.Sc. Thesis). University of KwaZulu-Natal, Pietermaritzburg.

BAJAJ, Y.P.S. and PIERIK, R.L.M. 1974. Vegetative propagation of *Freesia* through callus cultures. *Netherlands Journal of Agricultural Sciences* **22**: 153-159.

BAJAJ, Y.P.S., SIDHU, M.M.S. and GILL, A.P.S. 1983. Some factors affecting the *in vitro* propagation of *Gladiolus*. *Scientia Horticulturae* **18**: 269-275.

BEWLEY, J.D. and BLACK, M. 1985. Seeds. Physiology of Development and Germination. Plenum Press, New York. ISBN 0-306-41687-5

BRANTS, D.H. 1968. A revised medium for *Freesia* meristem culture. *Netherlands Journal of Plant Pathology* **74**: 120-121.

BROWN, D.C.W., LEUNG, D. W. M. and THORPE, T.A. 1979. Osmotic requirement for shoot formation in tobacco callus. *Physiologia Plantarum* **46**: 36-41.

CHICHIRICCO, G. and CAIOLA, M. 1987. *In vitro* development of parthenocarpic fruits of *Crocus sativus* L. *Plant Cell, Tissue and Organ culture* **11**: 75-78.

CORBINEAU, F. and CÔME, D. 1988. Storage of recalcitrant seeds of four tropical species. *Seed Science and Technology* **16**: 97-103.

CROUCH, I.J. and VAN STADEN, J. 1991. Evidence of rooting factors in a seaweed concentrate prepared from *Ecklonia maxima*. *Journal of Plant Physiology* **137**: 319-322.

DANTU, P.K. and BHOJWANI, S.S. 1995. *In vitro* corm formation and field evaluation of corm-derived plants of *Gladiolus*. *Scientia Horticulturae* **61**: 115-129.

DE BERGH, P. C. and READ, P. E. 1991. Micropropagation. In: De Bergh, P.C. and Zimmerman, R.H. (Eds.) Micropropagation: Technology and Application. Kluwer Academic, Dordrecht, The Netherlands. ISBN 0-7923-0818-2

DE BRUYN, M.H. and FERREIRA, D.I. 1992. *In vitro* corm production of *Gladiolus dalenii* and *G. tristis*. *Plant Cell, Tissue and Organ Culture* **31**: 123-128.

DELLAPORT, S.L., WOOD, J. and HICKS, J.B. 1985. Maize DNA miniprep. In: Malmberg, R., Messing, J. and Sussex, I. (Eds.) Molecular Biology of

plants. Cold Spring Harbour Laboratory Cold Spring Habour, New York. ISBN 0-87969-184-0

DONNELLY, D.J. and VIDAVER, W.E. 1988. Glossary of Plant Tissue Culture. Dioscorides Press, Portland Oregon. ISBN 0-931146-12-7

EDWARDS, N.R. 2000. Optimisation of the randomly amplified polymorphic DNA (RAPD) technique for the characterisation of selected South African maize (*Zea mays* L.) breeding material. (M.Sc. Thesis). University of Natal, Pietermaritzburg.

EL-GENDY, S.A., HASHIM, M.E., HOSNI, A.M. and LASHEEN, F.F. 2001a. *In vitro* shoot proliferation from *Gladiolus* from either terminal and lateral buds or segments of corms. *Arab Universities Journal of Agricultural Sciences* **9**: 889-913.

EL-GENDY, S.A., HASHIM, M.E., HOSNI, A.M. and LASHEEN, F.F. 2001b. *In vitro* cormel formation of *Gladiolus* cvs. 'Jackson Ville' and 'Peter Pears' and their *in vivo* germination. *Arab Universities Journal of Agricultural Sciences* **9**: 867-887.

ENGELMANN, F. 1998. *In vitro* conservation of horticultural genetic resources. Review of the state of the art. *Acta Horticulturae* **495**: 245-249.

ESTERHUIZEN, A.D., VAN DE VENTER, H.A. and ROBBERTSE, P.J. 1986. A preliminary study on seed germination of *Watsonia fourcadei*. *South African Journal of Botany* **52**: 221-225.

GEORGE, E.F. 1993. Plant propagation by tissue culture, Part 1, The Technology. Second edition. Exegetics Ltd., England. ISBN 0-9509325-4-X

GEORGE, E.F. 1996. Plant propagation by tissue culture, Part 2, The Practice. Second edition. Exegetics Ltd., England. ISBN 0-9509325-5-8

GOLDBLATT, P. 1971. Cytological and morphological studies in the southern Africa Iridaceae. *Journal of South African Botany* **37**: 317-460.

GOLDBLATT, P. 1990. Phylogeny and classification of Iridaceae. *Annals of the Missouri Botanical Garden*. **77**: 607-627.

HADRYS, H., BALICK, M. and SCHIERWATER, B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology* **1**: 55-63.

HILLIARD, O.M. and BURTT, B.L. 1991. Dierama-The Hairbells of Africa. Acorn Books CC, Johannesburg and London. ISBN 1-874802-01-7

HILLS, P.N. 2003. Differential gene expression in germinating and thermoinhibited achenes of *Tagetes minuta* L. (Ph.D. Thesis). University of Natal, Pietermaritzburg.

HILLS, P.N. and VAN STADEN, J. 2002. An improved DNA extraction procedure for plant tissue with a high phenolic content. *South African Journal of Botany* **68**: 549-550.

HOL, G.M.G.M. and VAN DER LINDE, P.C.G. 1992. Reduction of contamination in bulb-explant cultures of *Narcissus* by a hot-water treatment of parent bulbs. *Plant Cell, Tissue and Organ Culture* **31**: 75-79.

HOLLINGS, M.1965. Disease control through virus free stock. *Annual Review of Phytopathology* **3**: 367-396.

HUSSAIN, C.T.S., GEETHA, C.K., RAJEEVAN, P.K. and VALSALAKUMARI, P.K. 1997. Effect of triadimefon and sucrose on *in vitro* corm production in *Gladiolus* cv. 'Friendship'. *Journal of Ornamental Horticulture* **3**: 36-40.

HUSSEY, G. 1975b. Totipotency in tissue explants and callus of some members of the Liliaceae, Iridaceae, and Amaryllideceae. *Journal of Experimental Biology* **26**: 253-256.

HUSSEY, G. 1976c. *In vitro* release of axillary shoots from apical dominance in monocotyledonous plantlets. *Annals of Botany* **40**: 1323-1325.

HUSSEY, G. 1977a. *In vitro* propagation of some members of the Liliaceae, Iridaceae and Amaryllideace. *Acta Horticulturae* **78**: 303-309.

HUSSEY, G. 1977b. *In vitro* propagation of *Gladiolus* by precocious axillary shoot formation. *Scientia Horticulturae* **6**: 287-296.

JÄGER, A.K., McALISTER, B.J. and VAN STADEN, J. 1998. *In vitro* culture of *Gladiolus carneus. South African Journal of Botany* **64**: 146-147.

JEFFREYS, A.J., WILSON, V. and THEIN, S. L. 1985. Hypervariable minisatellite regions in human DNA. *Nature* **314**: 67-73.

JEPPE, B. 1999. Irises. Tien Wah Press (PTY) LTD. Singapore. ISBN 1-919766-11-1

KIM, C.S., LEE, C.H., SHIN, J.S., CHUN, Y.S. and HYUNG, N.I. 1997. A simple and rapid method for isolation of high quality genomic DNA from fruit trees and conifers using PVP. *Nucleic Acids Research* **25**: 1085-1087.

KIM, K. and DE HERTOGH, A.A. 1997. Tissue culture of ornamental flowering bulbs. *Horticultural Reviews* **18**: 87-147.

KOEN, K.J. 2001. Cultivation of *Combretum bracteosum* (Hochst.) Brandis. (Ph.D. Thesis). University of Natal, Pietermaritzburg.

LAURA, M., DE BENEDETTI, L., BRUNA, S., BURCHI, G., BERIO, T., GIOVANNINI, A. and RUFFONI, B. 2003. *Cyclamen persicum* Mill somatic embryogenesis and RAPD analysis of embryogenic callus. *Acta Horticulturae* **625**: 137-144.

LEE, M. 1995. DNA markers and plant breeding programmes. *Advances in Agronomy* **55**: 265-344.

LILIEN-KIPNIS, H. and KOCHBA, M. 1987. Mass propagation of new *Gladiolus* hybrids. *Acta Horticulturae* **212**: 631-638.

LILIEN-KIPNIS, H., STEIN, A. and LEVY, S. 1992a. The course of bean yellow virus elimination from *Gladiolus* by meristem tip culture. *Acta Horticulturae* **325**: 709-714.

LILIEN-KIPNIS, H., ZIV, M., KAHANY, S. and AZIZBEKOV, N. 1992. Proliferation and regeneration of *Nerine* in liquid cultures. *Acta Horticulturae* **325**: 467-474.

LINDSEY, K.L., JÄGER, A.K. and VAN STADEN, J. 1998. Effect of seaweed concentrate on acclimatization of *in vitro* grown plantlets of *Kniphofia pauciflora* and *Scilla krausii. South African Journal of Botany* **64**: 263-264.

LINSMAIER, E.M. and SKOOG, F. 1965. Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum* **18**: 100-127.

McALISTER, B.G., JÄGER, A.K. and VAN STADEN, J. 1998. The micropropagation of *Babiana* spp. *South African Journal of Botany* **64**: 88-90.

McDONALD, M.B. and COPELAND, L.O. 1989. Seed science and technology laboratory manual. Iowa State University Press, Iowa. ISBN 0-8138-0192-7

MEYER, H.J. and VAN STADEN, J. 1988. The *in vitro* culture of *Gerbera aurantica*. *Plant Cell, Tissue and Organ Culture* **14**: 25-30.

MILYAEVA, E.L., AZIZBEKOVAS, N., KOMAROVA, E.N. and AKHUNDOVA, D.D. 1995. *In vitro* formation of regenerant corms of saffron crocus (*Crocus sativus* L.). *Russian Journal of Plant Pathology* **42**: 112-119.

MOHAMED-YASEEN, Y. 2000. *In vitro* somatic embryogenesis and plant regeneration from *Gladiolus* root explants. *Annals of Agricultural Science (Cairo)* **45**: 647-657.

MUNTHALI, M.T., NEWBURY, H.J. and FORD-LLOYD, B.V. 1996. The detection of somaclonal variants of beet using RAPD. *Plant Cell Reports* **15**: 474-478.

MURASHIGE, T. 1974. Plant propagation through tissue cultures. *Annual Review of Plant Physiology* **25**: 135-166.

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

NEWBURY, H.J. and FORD-LLOYD, B.V. 1993. The use of RAPD for assessing variation in plants. *Plant Growth Regulation* **12**: 43-51.

NEWTON, C.R. and GRAHAM, A. 1994. PCR-polymerase chain reaction. Bios Scientific Publishers, Oxford. ISBN 1-87274-882-1

PAGE, Y.M. and VAN STADEN, J. 1985. *In vitro* propagation of *Dierama latifolium*. *HortScience* **20**: 1049-1050.

PAN, M.J. and VAN STADEN, J. 1998. The use of charcoal in *in vitro* culture-a review. *Journal of Plant Growth Regulation* **26**: 155-163.

PÂQUES, M., BERCETCHE, J. and DUMAS, E. 1992. Liquid media to improve and reduce the cost of *in vitro* conifer propagation. *Acta Horticulturae* **319**: 95-100.

PETRU, E., JIRSAKOVA, E. and LANDA, Z. 1976. Clonal propagation of some *Freesia* cultivars through tissue culture. *Biologia Plantarum* **18**: 304-306.

PIERIK, R.L.M. and STEEGMANS, H.M.M. 1975a. Effect of auxins, cytokinins, gibberellins, abscisic acid and ethepon on regeneration and growth of bulblets on excised bulb scale segments of hyacinth. *Plant Physiology* **34**: 14-17.

PIERIK, R.L.M. and STEEGMANS, H.M.M. 1976b. Vegetative propagation of *Freesia* through isolation of shoots *in vitro*. *Netherlands Journal of Agricultural Science* **24**: 274-277.

PIOLA, F., ROHR, R. and HEIZMANN, P. 1999. Rapid detection of genetic variation within and among *in vitro* propagated cedar (*Cedrus libani* Loundon) clones. *Plant Science* **141**: 159-163.

RAFALSKI, J.A. and TINGEY, S.V. 1993. Genetic diagnosis in plant breeding: RAPDs, microsatellites and machines. *Trends in Genetics* **9**: 275-280.

RAGOT, M. and HOISINGTON, D.A. 1993. Molecular markers for plant breeding: comparison of RFLP and RAPD genotyping costs. *Theoretical and Applied Genetics* **86**: 975-984.

RANI, V., PARIDA, A. and RAINA, S.N. 1995. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoids* Marsh. *Plant Cell Reports* **14**: 459-462.

RICHARDS, E.J. 1997. Preparation of plant DNA using CTAB. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and

Struhl, K. (Eds.) Short Protocols in Molecular Biology. 3rd edition. John Wiley and Sons, New York, pp 2.10-2.11. ISBN 0-471-13781-2

ROBERTS, E.H. 1973. Predicting the storage life of seeds. *Seed Science and Technology* **1**: 499-514.

SCOTT-SHAW, R. 1999. Rare and threatened plants of KwaZulu-Natal and neighbouring regions. Teeanem Printers, Pietermaritzburg. ISBN 0-620-24688-X

SHOYAMA, Y., ZHU, X.X., NAKAI, R., SHIRAISHI, S. and KOHDA, H. 1997. Micropropagation of *Panax notoginseng* by somatic embryogenesis and RAPD analysis of regenerated plantlets. *Plant Cell Reports* **16**: 450-453.

ŠIMKO, I. 1993. Effects of kinetin, paclobutrazol and their interactions on the microtuberization of potato stem segments cultured *in vitro* in the light. *Plant Growth Regulation* **12**: 23-27.

SIMONSEN, J. and HILDEBRANDT, A.C. 1971. *In vitro* growth and differentiation of *Gladiolus* plants from callus cultures. *Canadian Journal of Botany* **49**: 1817-1819.

STAFFORD, A. 1991. Genetics of cultured plant cells. In: Stafford, A. and Warren, G. (Eds.) Plant cell and tissue culture. Open University Press, London. ISBN 0-355-15832-4

STAFFORD, A. and WARREN, G. 1991. (Eds.) Plant cell and tissue culture. Open University Press, London. ISBN 0-355-15832-4

STEINITZ, B., COHEN, A., GOLDBERG, Z. and KOCHBA, M. 1991. Precocious *Gladiolus* corm formation in liquid shake cultures. *Plant Cell, Tissue and Organ Culture* **26**: 63-70.

STIMART, D.P. and ASCHER, P.D. 1978b. Propagation and stability of diploid *Freesia hybrida* cv. 'Royal' in tissue culture. *HortScience* **13**: 382-383.

STIMART, D.P. and ASCHER, P.D. 1982. Plantlet regeneration and stability from callus cultures from *Freesia* x *hybrida* Bailey cultivar 'Royal'. *Scientia Horticulturae* **17**: 153-157.

TAYLOR, J.L.S. 1998. An investigation into the biology and medicinal properties of *Eucomis* species. (Ph.D. Thesis). University of Natal, Pietermaritzburg.

TOWILL, L.E. 1988. Genetic considerations for germplasm preservation of clonal materials. *HortScience* **23**: 91-95.

UPFOLD, S.J., VAN STADEN, J. and EDWARDS, T.J. 1992. *In vitro* propagation of *Rhodohypoxis baurii*. *HortScience* **27**: 1230.

WAGNER, D.F. 1984. A techinique for demonstrating meristematic sites in bulbs and corms. *HortScience* **19**: 725.

WILLIAMS, J.K.G., KUBELIK, A.R., LIVAK, K.J. RAFALSKI, J.A. and TINGEY, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Research* **18**: 6531-6535.

WELSH, J. and McCLELLAND, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**: 7213-7218.

VAN AARTRIJK, J. and BLOM-BARNHOORN, G. J. 1980. Effects of sucrose, mineral salts and some organic substances on the adventitious regeneration *in vitro* of plantlets from bulb-scale tissue of *Lilium speciosum* 'Rubrum'. *Acta Horticulturae* **109**: 297-300.

VAN STADEN, J. and DAVEY, J.E. 1979. The synthesis, transport and metabolism of endogenous cytokinins. *Plant, Cell and Environment* **2**: 93-106.

VISHNEVETSKY, J., ZAMSKI, E. and ZIV, M. 2000. Carbohydrate metabolism in *Nerine samiensis* bulbs developing in liquid culture. *Physiologia Plantarum* **108**: 361-369.

VOS, P., HOGERS, R., BLEEKER, M., RAEIJANS, M., VAN DER LEE, T., HORNES, M., FRITERS, A., POT, J., PELEMAN, J., KUIPER, M. and ZABEAU, M. 1995. AFLP: A new concept for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4417.

ZHOU, P., HE, Y.K. and LI, S.J. 1999. Induction and characterization of *in vitro* corms of diploid-taro. *Plant Cell, Tissue and Organ Culture* **57**: 173-178.

ZIV, M., HALEVY, H. and SHILO, R. 1970. Organs and plantlets regeneration of *Gladiolus* through tissue culture. *Annals of Botany* **34**: 671-676.

ZIV, M. 1979. Transplanting *Gladiolus* plants propagated *in vitro*. *Scientia horticulturae* **11**: 257-260.

ZIV, M. 1989. Enhanced shoot and cormlet proliferation in liquid cultured *Gladiolus* buds by growth retardants. *Plant Cell, Tissue and Organ Culture* **17**: 101-110.

ZIV, M. 1990. The effect of growth retardants on shoot proliferation and morphogenesis in liquid cultured *Gladiolus* plants. *Acta Horticulturae* **280**: 207-214.

ZIV, M. and HADAR, A. 1991. Morphogenetic pattern of *Nephrolepis exalta* 'Bostoniensis' in agar or liquid cultures: Implications for micropropagation. *Israel Journal of Botany* **40**: 7-16.